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The Effect of Allopurinol (4-Hydroxypyrazolo(3,4-d) Pyrimidine) on Uric Acrid Metabolism in Differential Grasshoppers (Melanoplus Differentialis) and Armyworms (Pseudoletia Unipuncta)

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THE EFFECT OF ALLOPURINOL (4-HYDROXYPYRAZOLO(3,4-d)PYRIMIDINE) ON URIC ACID METABOLISM IN DIFFERENTIAL GRASSHOPPERS (MELANOPLUS DIFFERENTIALIS) AND ARMYWORMS (PSEUDOLETIA UNIPUNCTA)

BY

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This thesis is approved as a creditable and independent investigation by a candidate for the degree, Doctor of Philosophy, and is acceptable as meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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HCS

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Abstract

HENRY C. SCHROEDER, JR.

Under the supervision of Dr. Eugene W. Hamilton

Allopurinol, a potent inhibitor of xanthine oxidase in humans, was incorporated in the diets of differential grasshopper nymphs and adults and armyworm larvae. Uric acid contents of whole body homogenates decreased by highly significant rates. Feces uric acid decreased by highly significant rates in grasshopper nymphs and adults. Allopurinol also caused highly significant increases in mortality and inhibition of metamorphosis in study insects. These effects were not reversible by the addition of purine and pyrimidine bases, inosine, oxypurines, or uric acid. The effects were reversed by discontinuing application of allopurinol.

Crude xanthine oxidase, assayed by measuring the reduction of NAD at 340 mμ, from grasshoppers and armyworms was linear with respect to enzyme concentration. Allopurinol inhibition of xanthine oxidase was linear with respect to inhibitor concentration.

Kinetic studies on crude grasshopper xanthine oxidase were used to evaluate the Michaelis-Menten constant for the oxidation of hypoxanthine ($K_m = 2.52 \times 10^{-5}$ M), and to study xanthine oxidase inhibition (mixed) by allopurinol ($K_i = 3.95 \times 10^{-5}$ M).

Abstract (Cont.)

Electron microscopy of Malpighian tubule sections from grasshopper nymphs treated with allopurinol showed a reduction in size and number of vacuoles as compared to a control.

INTRODUCTION

Terrestrial insects and man use xanthine oxidase to convert hypoxanthine to xanthine and xanthine to uric acid (Anderson and Patton 1955, Hundles et al. 1966). Insects use uric acid, a metabolic breakdown product of purines, as their major nitrogenous excretory product (Rockstein 1965). The enzymes and intermediate products needed in the production of uric acid have been identified in mammals and insects (Anderson and Patton 1955, Cantarow and Schepartz 1967). The in vivo inhibition of uric acid production has not been reported in insects.

The purine analogue allopurinol (4-hydroxypyrazolo(3,4-d)pyrimidine) inhibits the enzyme xanthine oxidase and ultimately uric acid production (Webb 1963). Burroughs Wellcome and Company (U.S.A.), Incorporated, Tuckahoe, New York produces 'Zyloprim' brand allopurinol in 100 mg scored tablets for clinical use in the treatment of gout, a condition in man of overproduction or underexcretion of uric acid (Anonymous 1967). I became interested in allopurinol in 1968 after running an exploratory feeding experiment using house crickets, Acheta domestica (Linn.). Uric acid levels decreased significantly in allopurinol fed crickets and also caused a marked increase in mortality.

The objectives of further studies were to investigate the effects of the decrease in uric acid levels and the increased mortality as applied to purine catabolism and uric acid excretion in the differential grasshopper, Melanoplus differentialis (Thomas) and the armyworm, Pseudoletia unipuncta (Haworth).

LITERATURE REVIEW

As in birds and certain reptiles, 50 to 80% of the total nitrogen of terrestrial insect excreta is uric acid (Rockstein 1965). Nation and Patton (1961) and Nation (1963) reported on the examination of feces of all stages of Acheta domestica (L.), Oncopeltus fasciatus (Dallus), Periplaneta americana (L.), Galleria mellonella (L.), and the larvae of Tenebrio molitor (L.) and Malacosoma americana (F.) for ademine, quanine, uracil, thymine, cytosine, hypoxanthine, xanthine, allantoin, urea, and uric acid. Uric acid was the major nitrogenous compound excreted in all these insects. T. molitor excreted some urea and G. mellonella larvae excreted some hypoxanthine and xanthine in addition to uric acid.

Three species of adult mosquitoes Aedes aegypti (L.), Anopheles quadromaculatus (Say), and Culex pipians (L.) excrete 50% of their total nitrogenous excreta as uric acid; urea (10%), ammonia (10%), amino acid (5%), and protein nitrogen (10%) account for an additional 35% of the total nitrogen in the adult mosquitoes (Irreverre and Terzian 1959).

Internal uric acid levels have been reported for haemolymph and fat bodies of larvae of the last instar of Prodenia eridania (Cramer) and G. mellonella, and adults of P. americana (Anderson and Patton 1955). Blood uric acid of Prodenia larvae was found to be up to 2.6 times as high in fed insects (22.3 $\pm$ 3.8 mg% uric acid) as in those that have been starved (8.7 $\pm$ 4.6 mg% uric acid). The haemolymph of

fed G. mellonella larvae and P. americana nymphs contained 8.0 ± 4.7 and 4.4 ± 1.9 mg% uric acid respectively. The fat bodies of adult P. americana contained 43.3 ± 19.9 mg% uric acid when fed a normal diet and 156.7 ± 48.9 mg% uric acid when starved. The feeding of a xanthine enriched diet had no significant effect on uric acid production in P. americana. Anderson and Patton also incubated fat bodies of P. americana with various purines and found a highly significant increase in uric acid levels, demonstrating the presence of guanase, adenase, and xanthine oxidase in the fat bodies.

Barrett and Friend (1970) used ^{14}C-labeled glycine-1, glycine-2, and sodium formate to investigate the origin of the carbon atoms of uric acid in Rodnius prolixus (L.). The results demonstrated that the carboxyl carbon of glycine was incorporated into position 4, the alpha carbon of glycine in position 5, and the carbon of sodium formate in positions 2 and 8 of uric acid. These results agreed with those reported for other uricotelic organisms.

Irzykiewicz (1955) studied xanthine oxidase activity in tissue homogenates of Tineola bisselliella (Hummel), T. molitor, Lucilia cuprinia (Wied.), Anthrenocerus australis (Hope), Ephestria kuhniella (Zell.), and Anthrenus flavipes (LeConte). He reported that the activity of this enzyme in Tineola larvae averaged 200 µM of uric acid per gram whole larvae (wet weight) per hour. The activity of xanthine oxidase in the other species studied ranged from 15.4 to 1.3 µM of uric acid per gram whole larvae per hour. The optimum pH for Tineola xanthine oxidase activity was shown to be

between pH 7.7 and 8.0.

The activity of xanthine oxidase in cultured embryonic cells of Drosphila melanogaster (Meigen) was studied by Horikawa et al. (1967) for the effects of the substrates hypoxanthine, xanthine, and uric acid on enzyme levels. Cells incubated with varying substrate concentration lowered the specific activity of xanthine oxidase. This decrease in activity was attributed to the conversion of hypoxanthine → xanthine → uric acid and inhibition of xanthine oxidase by the resulting uric acid. They also found uric acid to be a powerful noncompetitive inhibitor of the enzyme in whole larval homogenates of D. melanogaster. Uric acid concentrations of 4×10^{-4}M and 3.2×10^{-3}M inhibited larval xanthine oxidase activity 41.6 and 79.2%, respectively. Inhibition of xanthine oxidase was reversed by dialysis. They also found the dissociation constant, Ki, for the enzyme-inhibitor complex to be $2.19 \pm 0.03 \times 10^{-4}$M.

The solubility of uric acid in water at 37° C is 6.45 mg%. Concentrations over this amount result in supersaturation. Supersaturation of insect blood is very common but no pathological deposition of urate crystals have been described. In mammals, supersaturation (above 8 mg%) generally results in hyperuricemia and hyperuricosuria with the deposition of urate crystals or tophaceous nodules. These nodules consist of multiconcentric deposits of amorphous or crystalline urates in a radial matrix composed of polysaccharides, lipids, protein, and occasionally calcium (Sokoloff 1957). One common manifestation of hyperuremic conditions

in humans is gout which results from overproduction or underexcretion of uric acid (Sorensen 1963).

The compound 4-hydroxypyrazolo(3,4-d)pyrimidine (4-HPP) along with analogues of other purines were originally synthesized as antitumor agents (Robins 1956, Falco and Hitchings 1956). Most of these compounds were found ineffective for the purpose intended. Several of the purine analogues tested in vivo were found to be very potent xanthine oxidase inhibitors (Feigelson et al. 1957). Analysis of preliminary data on laboratory animals, demonstrated 4-HPP to be relatively non-toxic and prompted the selection of 4-HPP for an in vivo experiment designed to test it as an adjuvant to the antileukemic action of 6-mercaptopurine (6-MP) (Elion et al. 1954). They administered both 6-MP and 4-HPP to mice and found that 6-MP was not as extensively oxidized to 6-thiouric acid by xanthine oxidase as when only 6-MP was used.

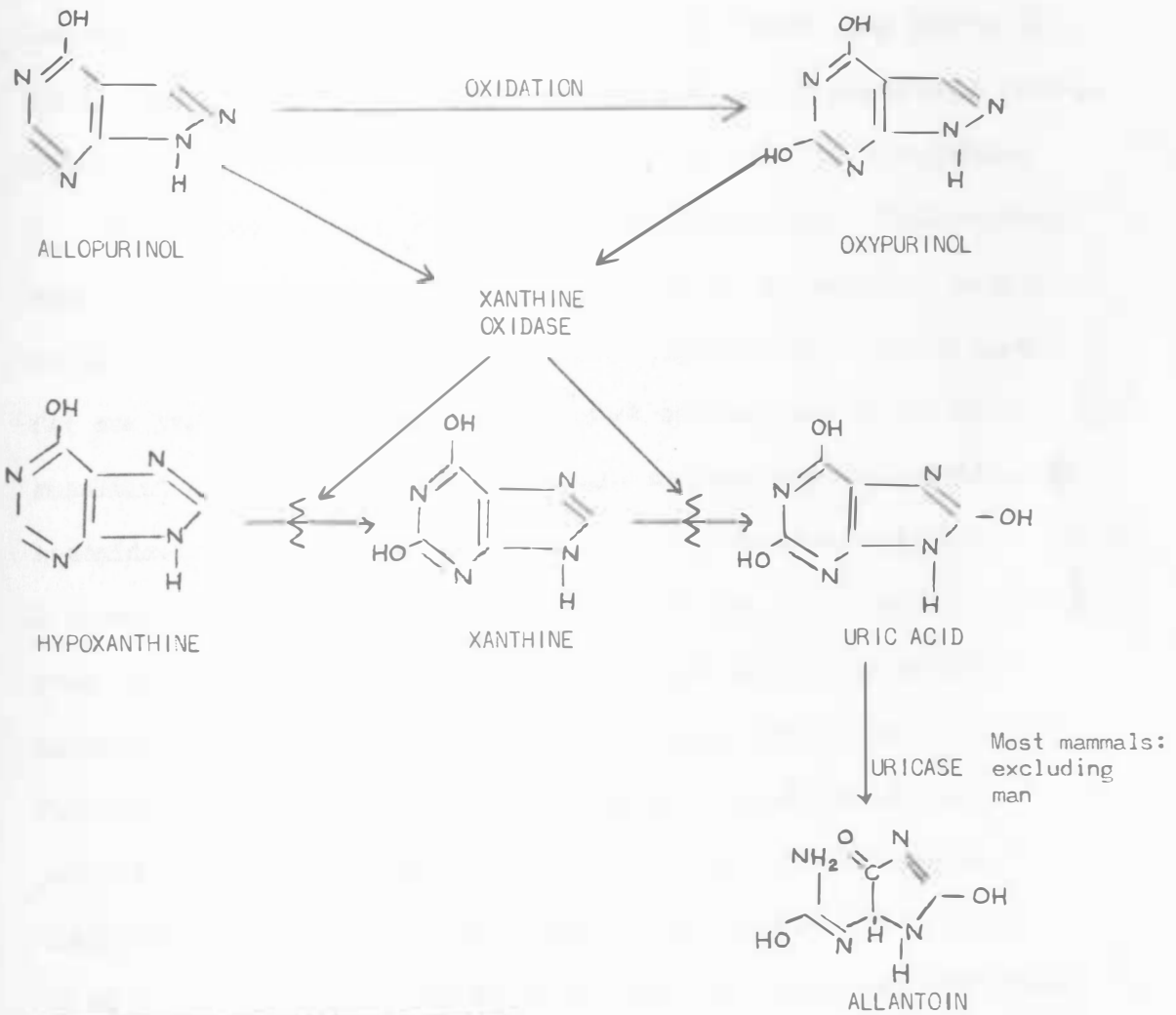
A decline in both serum and urinary uric acid levels was noted in human patients with leukemia and hyperuricemia treated with 4-HPP and 6-MP (Rundles et al. 1963). These results prompted further trials of the use of 4-HPP in treatment for primary gout. Striking reductions in serum and urinary uric acid levels along with increases in hypoxanthine and xanthine occurred in patients treated with 4-HPP (Elion et al. 1963). Very few side reactions were noted. Subsequently, 4-HPP was given the generic name of allopurinol. Allopurinol has found extensive use in treatment of hyperuricemia and hyperuricosurea of varying etiology.

Allopurinol is a structural analogue of the natural purine base, hypoxanthine, and differs by transposition of nitrogen and carbon atoms in positions 7 and 8 (Fig. 1). It acts as a substrate for and potent competitive inhibitor of xanthine oxidase ($Ki = 3.2 \times 10^{-5}$mM) being bound 15 times more tightly to the enzyme than is xanthine (Elion et al. 1963). The compound formed by xanthine oxidase catalyzed oxidation of allopurinol is the xanthine analogue, oxypurinol or alloxanthine (4,6-dihydroxypyrazolo(3,4-d)pyrimidine). Oxypurinol is also an inhibitor of xanthine oxidase but less active than allopurinol ($Ki = 5.4 \times 10^{-5}$mM).

The two metabolic steps leading to uric acid formation in mammals are undoubtedly the main points of action of allopurinol inhibition *in vitro* and *in vivo* (Rundles et al. 1963 and 1964, Elion et al. 1963, Wyngaarden et al. 1963, Klinenberg et al. 1963 and 1965, Rundles et al. 1964, Yu and Gutman 1964).

McCollister et al. (1964) has shown that 4-hydroxypyrazolo(3,4-d) pyrimidine ribonucleotide inhibits avian glutamine ribosylpyrophosphate-5-phosphate amidotransferase, the enzyme that catalyzes the first step in *de novo* synthesis of purines. When mice were fed both oxypurines and allopurinol, xanthine oxidase was inhibited and utilization of hypoxanthine and xanthine was increased (Pomales et al. 1963). The mice fed allopurinol incorporated oxypurines into nucleic acids. When allopurinol was omitted from the diet the oxypurines were catabolized to uric acid. Alexander et al. (1966) administered allopurinol to rats in standard diet and also as an intraperitoneal injection but

Fig. 1. Chemical structures and relationships between allopu
oxypurinol and terminal compounds in the biosynthesis of uric
(Thibodeau et al. 1969).


OH
N
N
N
H
ALLOPURINOL
OXIDATION
OH
HO
N
N
N
H
OXYPURINOL
XANTHINE
OXIDASE
OH
N
N
N
H
HYPOXANTHINE
OH
HO
N
N
N
H
XANTHINE
OH
HO
N
N
N
H
OH
URIC ACID
URICASE
Most mammals:
excluding
man
O
NH2
C
N
H
N
H
HO
OH
ALLANTOIN
= Inhibition

found no cumulative effect of the compound. A decrease in xanthine oxidase activity and inhibition of purine biosynthesis in minced rat liver was determined by measuring the fixation of ^{14}C-labeled formate in vitro. The following compounds decreased (as a percent of the control): inosine (59%), adenosine (91%), A5P (82%), ADP (83%), ATP (62%), NAD (78%), and GMP (69%). Concentrations of malic acid (209%), citric acid (151%), glutamic acid (151%), and IMP (161) increased.

Allopurinol is only moderately toxic to mammals. Dogs survived oral feeding at 30 mg/kg/day for one year with nil to minor changes in the kidney and no other significant abnormalities. At 90 mg/kg/day for one year there was some accumulation of xanthine in the kidneys with resultant chronic irritation and slight tubular changes. Occasional hemosiderin-like deposits were seen in the reticulo-endothelial system. A higher dose (270 mg/kg/day) resulted in large concentrations in the renal pelves, with severe destruction in the kidney secondary to xanthine accumulation. The deposition of xanthine appears to be a function both of the metabolic turnover of purines (which is proportionately larger in the smaller animals) and the degree of inhibition of xanthine oxidase. The average dosage for an adult 70 kg patient is 200 to 300 mg allopurinol per day (Anonymous 1967).

MATERIALS AND METHODS

Exploratory cricket experiment.--Forty male house crickets (Gryllous domesticus) of a shipment obtained for an insect physiology class, were divided into 2 groups of 20 each. One group received a diet of only turkey starter passed through a 36-mesh screen. The other group received the same diet plus 25 mg of allopurinol per gram of diet. Water was supplied by capillary action in a cellulose sponge. The crickets were allowed to feed on the diet for 10 days and the crickets remaining alive were sacrificed by freezing in a refrigerator-freezer compartment and then in liquid nitrogen. The crickets were ground and the residue dried in a vacuum oven, weighed, and suspended in equal volumes of distilled water on a weight basis. The water was heated to 80° C to bring the uric acid into solution and then filtered through a Whatman No. 1 filter. Colorimetric uric acid analysis on the filtrates was determined by standard hospital procedures at the Municipal Hospital, Brookings, South Dakota.

Grasshopper rearing.--Grasshopper eggs were oviposited in 5 X 10 X 3 inch plastic pans filled with moist sand. The pans were collected once a week from cages containing about 100 adult grasshoppers. The egg pods were transferred to a plastic container filled with $\frac{1}{2}$ inch of moist sand, an inch of moist sand added and the box covered for refrigeration. Diapause was broken by storing the eggs for a minimum of 3 months at 3 to 5° C. Eggs could be stored for an extended period under refrigeration.

Hatching was induced by removing the eggs from refrigeration and

incubating them for 19 to 25 days at 75° F and 60% humidity. The sand containing the eggs was kept moist but not wet during incubation. After 17 or 18 days of incubation, egg containers were placed in a small cage covered with fine mesh plastic screen. One side of the cage had a flannel sleeve for entrance into the cage. Newly-hatched nymphs were fed an artificial diet (Sutter and Miller 1971) cut into pieces about 1 inch square. Diet was replace d every 2 days. This diet dried out rather rapidly unless the humidity in the chamber was kept at about 70 to 75% humidity. It could be made up in large batches and stored under refrigeration for a week. Formalin placed in the diet eliminated most of the disease problems commonly associated with grasshoppers.

Nymphs took 2 months to reach adulthood. Adults were fed corn plants since the artificial diet lacked some nutrient needed for egg production. Grasshoppers began laying eggs 2 to 3 weeks after molting into adults.

Grasshopper and armyworm experiments in vivo.—Experiment A. Three hundred newly-molted-adult grasshoppers were divided into 5 groups of 60 and placed in 5 cages numbered 1 through 5. The cages were 1 foot square, wood framed, and covered with wire screen. The floor of each cage was lined with aluminum foil to collect feces. Cages were held under continuous fluorescent lighting for the duration of the experiment.

All adults were fed artificial diet for 2 days prior to treatment. The third day, grasshoppers in cages numbered 1, 2, 3, and 4 received

25,000, 15,000, 10,000, and 5,000 ppm allopurinol, respectively, in their diets as follows: each allopurinol dose was stirred into 100 ml aliquots of liquid diet held in a 60° C water bath; the prepared diet was poured into 25 ml plastic cups (4 cups/dose); and refrigerated. Allopurinol was in the form of ground 100 mg tablets[1] (100 mg allopurinol/0.3088 grams ground tablet). A cup of treated diet was fed to the grasshoppers each day. The control grasshoppers in cage No. 5 received only artificial diet.

Grasshoppers were removed for chemical analysis in groups of 5. The following schedule was followed: day 3 one group was removed, day 5 and day 8 three groups were removed, and day 10 the remaining 5 groups were removed from each cage.

Each group of grasshoppers was weighed, frozen to-60° C, and stored at -30° C. The frozen samples were subsequently freeze-dried, weighed, ground in a mortar and pestle, and refrozen until they could be chemically analyzed.

Feces samples were collected by vacuum from the cage foil every 2 days. These samples were frozen at -30° C, freeze-dried, homogenized in a Wig-L-Bug® (Hamilton 1967) and stored at -30° C for later chemical analysis.

Experiment 3.--First-instar-grasshopper nymphs two weeks old were divided into 3 groups. Lot No. 1 (100 nymphs) was fed diet

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[1]Zyloprim brand allopurinol, Burroughs Wellcome & Co. (U.S.A.), Inc. Tuckahoe, New York and Burlingame, California.

containing 6,476 ppm allopurinol in ground tablet form. Lot No. 2 (100 nymphs) was fed diet for 16 days and on the 17th day, 6,476 ppm allopurinol was added to their diet. Lot No. 3 (150 nymphs) received only untreated diet. Mortality was checked daily in all cages and the dead nymphs removed and the number recorded. Feces samples were collected at various intervals posttreatment from each lot.

Grasshoppers from lots No. 1 and 2 were sacrificed after 22 and 47 days, respectively. An equal number from lot No. 3 was sacrificed at the same time. The grasshopper nymphs and their feces samples were handled as described for adult grasshoppers.

Experiment C.--Newly-hatched-armyworm larvae obtained from the Northern Grain Insect Research Laboratory were placed on artificial diet (Sutter and Miller 1971) and held for 2 days. Two larvae were placed in each 25 ml plastic cup containing 5 ml of artificial diet. Treatments No. 1 through 4 (65 cups per treatment) received the same diet treated with 6,476, 3,138, 1,569, and 623 ppm allopurinol. Treatment No. 5 (65 cups) received untreated diet. Allopurinol was in the form of ground 100 mg tablets (100 mg allopurinol/0.3088 grams ground tablets) and mixed in the same manner as in the grasshopper experiment.

One hundred larvae from each treatment were observed for 27 days, and the following recorded: mortality, pupation, and weights of 3 lots of 15 larvae from each treatment at 12 days postemergence. From the other 30 larvae in each treatment, 3 lots of 5 larvae were weighed at 16 days postemergence, frozen at -60° C, freeze-dried, weighed, and refrozen at -30° C until they could be chemically analyzed.

Experiment D.—This experiment was the same as above except pure allopurinol[2] was used instead of the ground tablets. The larvae remaining at the end of 27 days (treatment No. 1) were weighed and placed on untreated diet to note if the effects of allopurinol were reversible. The latter were checked for mortality, pupation, emergence, and morphology.

Experiment E.—Newly-hatched-armyworm larvae were handled as in experiment C. Purines were added to the diets of 5 treatments of 50 larvae per treatment in the following concentrations: No. 1, nothing extra added; No. 2, 0.001 M uric acid; No. 3, 0.001 M of each adenine, guanine, thymine, cytosine, and uracil; No. 4, 0.001 M hypoxanthine and xanthine; and No. 5, 0.001 M inosine. Each of the 5 treatments was run in duplicate groups of 50 larvae, one group had allopurinol added (1,569 ppm) in addition to the purines and was omitted from the second group. The larvae, from each group of 50, were weighed in 3 aggregations of 14 larvae at 12 and 16 days postemergence. Mortality and pupation were also noted at this time.

Uric acid analysis.—Frozen armyworm, grasshopper, and feces samples were allowed to come to room temperature before opening the containers. Two-tenths gram of a sample was weighed, placed in a 15-ml-screw-top test tube, and 10 ml of 0.4% NaOH added. The tube was agitated for 2 minutes on a Vortex® test tube mixer and allowed to stand overnight at room temperature. The tube contents were mixed

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[2]Furnished by Burroughs Wellcome & Co. (U.S.A.).

again and filtered through glass fiber filters[3] using vacuum.

Uric acid levels were determined on the filtrate using a modification of the procedure of Praetorius and Poulsen (1953). All reagents were obtained commercially[4]. The procedure is based on the conversion of uric acid (high OD_{292}) to allantoin (low OD_{292}) by the enzyme uricase. The method follows:

1. Pipette into a test tube:

   0.2 ml filtrate

   1.0 ml glycine buffer

   6.0 ml distilled water (mix well by shaking)

2. Pipette into each of two cuvettes 3.0 ml of mixture from Step (1) - Label one Blank and the other Test

3. Pipette into the Test:

   0.05 ml uricase

   Pipette into the Blank:

   0.05 ml distilled water

   Allow both to stand at room temperature for 30 minutes.

4. a) Adjust spectrophotometer to wavelength of 292 mu.

   b) Adjust slit width so that OD_{292} of Blank reads 0.4000.

   c) Read and record OD of Test vs Blank.

5. After approximately 5 minutes:

   a) Again adjust slit width so that Blank reads 0.400.

   b) Read and record OD of Test. If OD has not changed, this is considered as a "Final OD". If it has decreased,

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[3] Reeve angel glass fiber filter 934 AH.

[4] Sigma Chemical Co., St. Louis, Missouri.

repeat readings at intervals until OD is constant ("Final OD"). Time required is not critical.

Calculations:

1. Since uricase is not used in the Blank, the small OD it contributes is approximately corrected for by adding 0.005 to the change in OD (Sigma uricase has been found to yield approximately this OD).

2. $\Delta OD = (0.400 - \text{final OD}) + 0.005$

   $= (0.405 - \text{final OD})$

3. Mg uric acid per 10 ml of filtrate.

$$= \frac{(3.05) \times (168.1) \times (10) \times (\Delta OD)}{(12,300) \times (0.0833)}$$

$$= (5) \times (\Delta OD)$$

Factors Used in Calculations:

1. 3.05 is the volume of reaction mixture.
2. 168.1 is the molecular weight of uric acid.
3. 12,300 is the OD of 1 mole of uric acid in 1 liter.
4. 10 converts 1 ml of filtrate to 10 ml.
5. 0.0833 is the volume of filtrate used.
6. 0.005 is the OD contributed by uricase.

In vitro experiments.--Ten adult grasshoppers were weighed after removal of their wings and legs. All extraction procedures were carried out in a cold room at 3 to 4° C. The grasshoppers were homogenized 5 each in Ten-Brock homogenizers adding 1 ml of buffer (0.1 M Tris, pH 7.9) per grasshopper and 1 ml of buffer rinse per 5 grasshoppers. One gram of cold purified Norit-A (Parzen and Fox

1964) was mixed with the combined homogenates (16 to 17 ml) and allowed to stand for 1 hour with occasional stirring. The mixture was centrifuged at 30,000 X gravity for 30 minutes and filtered through a sintered glass filter to remove any excess charcoal. The filtrate (9.5 to 10 ml) was used for enzyme analysis. Last-instar armyworm larvae were extracted in the same manner.

Xanthine oxidase activity was assayed by an adaptation of the method of Horikawa et al. (1967). In the presence of enzyme, 2 moles of NAD (nicotinamide-adenine dinucleotide) were reduced to 2 moles of NADH + H + during the oxidation of hypoxanthine→ xanthine→ uric acid. Enzyme, NAD, and buffer were allowed to equilibrate for 15 minutes and hypoxanthine added to begin the reaction. NAD reduction was measured at 340 mu on a Hatachi-Perkin Elmer® 139 UV-VIS spectrophotometer, using 1 cm quartz cuvettes, and recorded on a Beckman® 10-inch recorder. Absorbance does not increase in the absence of either NAD or hypoxanthine. A unit of enzyme activity (V) was defined as a change of 1.0% transmittance (ΔT) over a period of 5 minutes. Specific activity was defined as the number of units of enzyme activity per mg protein. Protein was determined by Goa's microbiuret method with crystalline bovine serum albumin as a standard (Bailey 1962).

Standard enzyme activity was measured at room temperature in mixtures with a total volume of 3.0 ml containing 0.2 ml of enzyme extract, 0.2 ml of 5.25×10^{-2} M NAD, 0.2 ml of 2.25×10^{-3} M hypoxanthine, and 2.4 ml of 0.1 M Tris buffer (pH 7.9). All reagents were made using 0.1 M Tris buffer (pH 7.9). Blanks contained 0.2 ml

of enzyme extract, 0.2 ml of NAD, and 2.6 ml of buffer.

Variations from the standard assay for grasshopper xanthine oxidase activity were run as follows: (1) the concentration of enzyme was varied keeping the volume and other ingredients constant, (2) 0.2 ml of various dilutions of allopurinol were added keeping other factors constant, (3) 0.2 ml of enzyme extract was assayed at 4 different hypoxanthine concentrations, (4) 0.2 ml of enzyme extract plus 0.2 ml of 1.35×10^{-5} M allopurinol was assayed at each of 3 different hypoxanthine concentrations (Table 1, tests 1 - 4). Blanks were run with each reaction. All reactions were repeated 3 or more times and the results averaged.

Two tenths of a milliliter of enzyme extract obtained from last-instar armyworm larvae was assayed for activity. It was also assayed with several dilutions of allopurinol (Table 1, test 5).

Electron microscopy.—Twenty-five 2nd-instar-grasshopper nymphs were placed on artificial diet containing 6,476 ppm allopurinol in ground tablet form for 20 days. The digestive tracts, with Malpighian tubules attached, of these nymphs were removed and placed in fixative (cold 2.5% gluteraldehyde plus 0.2% thioglycollic acid mixed in 0.05 M K_2PO_4 buffer pH 7.0) for 12 hours (Langenberg and Schroeder 1970). Excess fixative was removed with 3 thirty-minute washings of cold buffer and one overnight washing. The tract was post-fixed in 1% OsO_4 in 0.05 M K_2PO_4 buffer (pH 7.0) 12 hours and dehydrated by transferring the tracts through the following cold acetone washes: 25% for 10 minutes, 50% for 24 hours, 75% for 30 minutes, 100% for

Table 1.--Concentrations of reactants [a] in enzyme assays.

Test		Hypoxanthine [S]	Enzyme ml	Allopurinol [I]	ΔT/ 5 min. [b] V
I.	(a)	1.5×10^{-4}M	0.1	none	18.70
	(b)	"	0.15	"	14.10
	(c)	"	0.2	"	9.83
II.	(a)	1.5×10^{-4}M	0.2	0.225×10^{-6}M	18.70
	(b)	"	"	0.450 "	16.60
	(c)	"	"	9.000 "	9.80
	(d)	"	"	11.250 "	8.30
	(e)	"	"	22.500 "	2.00
	(f)	"	"	35.600 "	0.00
III.	(a)	1.5×10^{-4}M	0.2	none	18.70
	(b)	0.750 "	"	"	16.00
	(c)	0.375 "	"	"	13.00
	(d)	0.150 "	"	"	8.20
IV.	(a)	1.5×10^{-4}M	0.2	9.0×10^{-6}M	9.80
	(b)	0.5 "	"	"	6.70
	(c)	0.3 "	"	"	5.40
V.	(a)	1.5×10^{-4}M	0.2	None	8.70
	(b)	"	"	12.50×10^{-6}M	7.50
	(c)	"	"	25.00 "	6.20
	(d)	"	"	50.00 "	3.10

[a] NAD concentration was 3.5×10^{-3}M in all reactions.

[b] Average of 3 or more reactions.

30 minutes, and another 100% for 1 hour. Controls were fixed in the same manner.

Imbedding plastic was made by mixing the following: 50 ml Araldite-6005, 16 ml Epon-812, 75 ml DDSA (dodecenylsuccinic anhydride), and 1.4 ml DMP-30 (tridimethylaminomethyl phenol). The digestive tracts were placed in 1:1 acetone:plastic for 4 to 6 hours, the last hour at 40° C. Malpighian tubules were dissected from the tract while in the mixture. These tubules were imbedded by placing them in BEEM® capsules containing 100% plastic. The plastic was polymerized at 60° C for 24 hours. Thin sections were cut on a Sorval Porter-Blum MT2 microtome® and mounted on 300 mesh copper grids. Sections were stained for 2 hours with 2% uranyl acetate, washed in distilled water, dried, stained 2 minutes in lead citrate solution in a CO_2 free atmosphere, washed in cool freshly boiled water, and dried. The sections were examined in an RCA-EMU3® electron microscope.

Statistical analysis.—Analysis of variance, t-test comparing adjacent means, Dunnett's test, and Chi-square tests were applied at the 99% and 95% level of confidence to determine if the data was highly significant or significant (Steel and Torrie 1960).

RESULTS

Exploratory experiment.—Uric acid levels in house crickets fed 25 mg allopurinol per gram of turkey starter for 10 days decreased 39.33%. Filtrates from treated crickets contained 54 mg% and untreated 89 mg% uric acid. There was 55% and 15% mortality in treated and untreated crickets respectively.

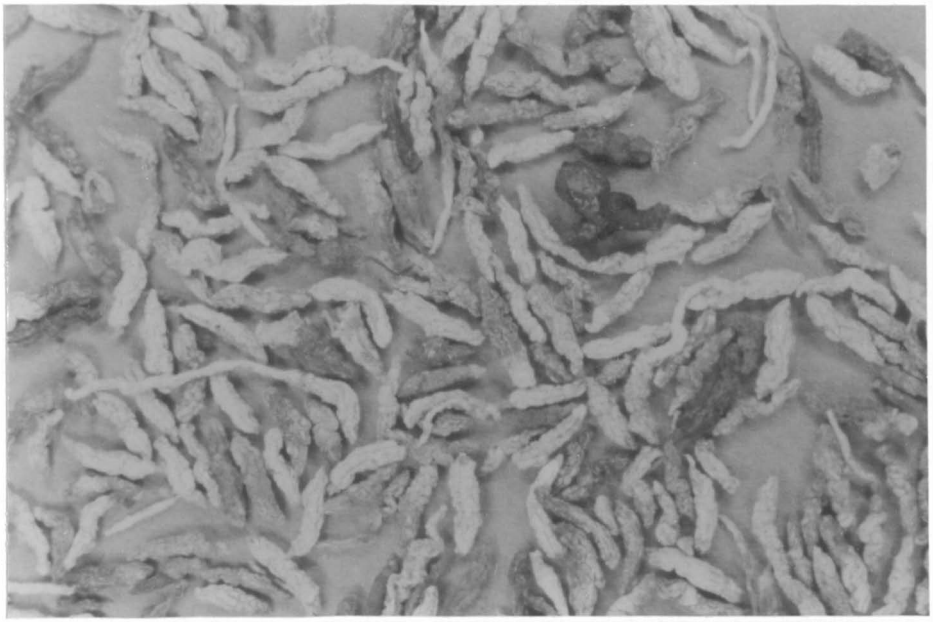
Grasshopper and armyworm experiments *in vivo*.—Experiment A. Adult grasshoppers fed allopurinol treated diet for 10 days showed no noticeable signs of distress and only one died. They consumed 174.23, 174.06, 180.34, 180.23, and 219.18 grams of diet from highest to lowest dose of allopurinol and control respectively. Morphology of feces from treated grasshoppers (Fig. 2) was markedly different from untreated grasshoppers (Fig. 3). The total amount of feces excreted by treated grasshoppers, as measured in vials of equal volumes was less than that of controls.

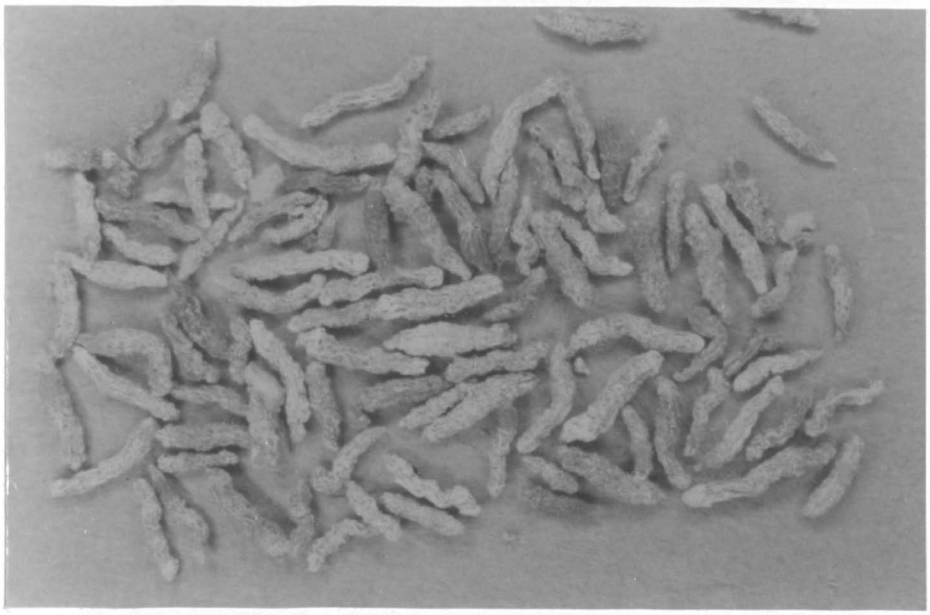
Body uric acid showed a highly significant decrease with time posttreatment, between treatment levels, and from day 0 to day 10 with a nonsignificant plateau between days 2 and 5. Control grasshopper uric acid decreased significantly from day 0 to day 2 but did not decrease significantly thereafter (Fig. 4). Body uric acid of treated grasshoppers decreased 63.22%, 51.52%, 70.88%, and 46.85% from the highest to lowest dose of allopurinol and the control 31.52% at 10 days posttreatment. Body uric acid of control grasshoppers averaged 3.035 mg/gram dry weight.

Fecal uric acid exhibited a highly significant difference in levels

Figure 2. Feces from adult grasshoppers fed artificial diet treated with 25,000 ppm allopurinol.

Figure 3. Feces from adult grasshoppers fed aritificial diet only.





between the length of the treatment period and also between treatment doses. The fecal uric acid of allopurinol treated grasshoppers decreased at a highly significant rate from day 0 to day 4 but there was no significant change from day 4 to day 10. Fecal uric acid of controls increased at a highly significant rate from day 0 to day 8 but not after that (Fig. 5). There was 90% or more reduction in fecal uric acid from treated grasshoppers during the treatment period while control levels increased 30.17%. Fecal uric acid contents of control grasshoppers averaged 51.15 mg/gram dry weight.

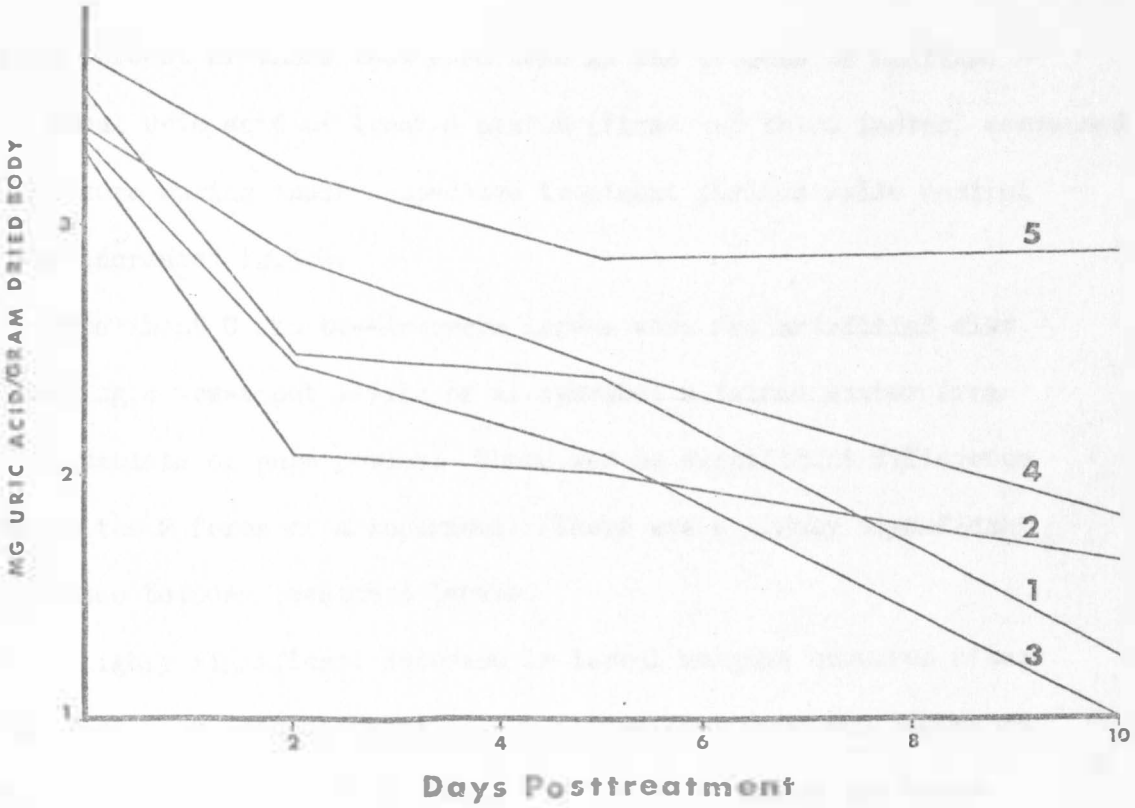
Experiment B.--First-instar-grasshopper nymphs fed 6,476 ppm allopurinol in their diets for 22 days suffered 86% mortality. At 22 days the remaining nymphs were weighed (average 0.330 grams wet and 0.123 grams dry weight/7 nymphs) and compared to an equal number of controls (average 3.49 grams wet and 1.07 grams dry weight/7 nymphs). There was a difference of 88.55% in dry weights. Uric acid in treated nymphs (1.40 mg/gram dry weight) and untreated nymphs (3.35 mg/gram dry weight) differed by 58.21%. None of the treated nymphs reached the third instar while all of the controls survived through the adult stage.

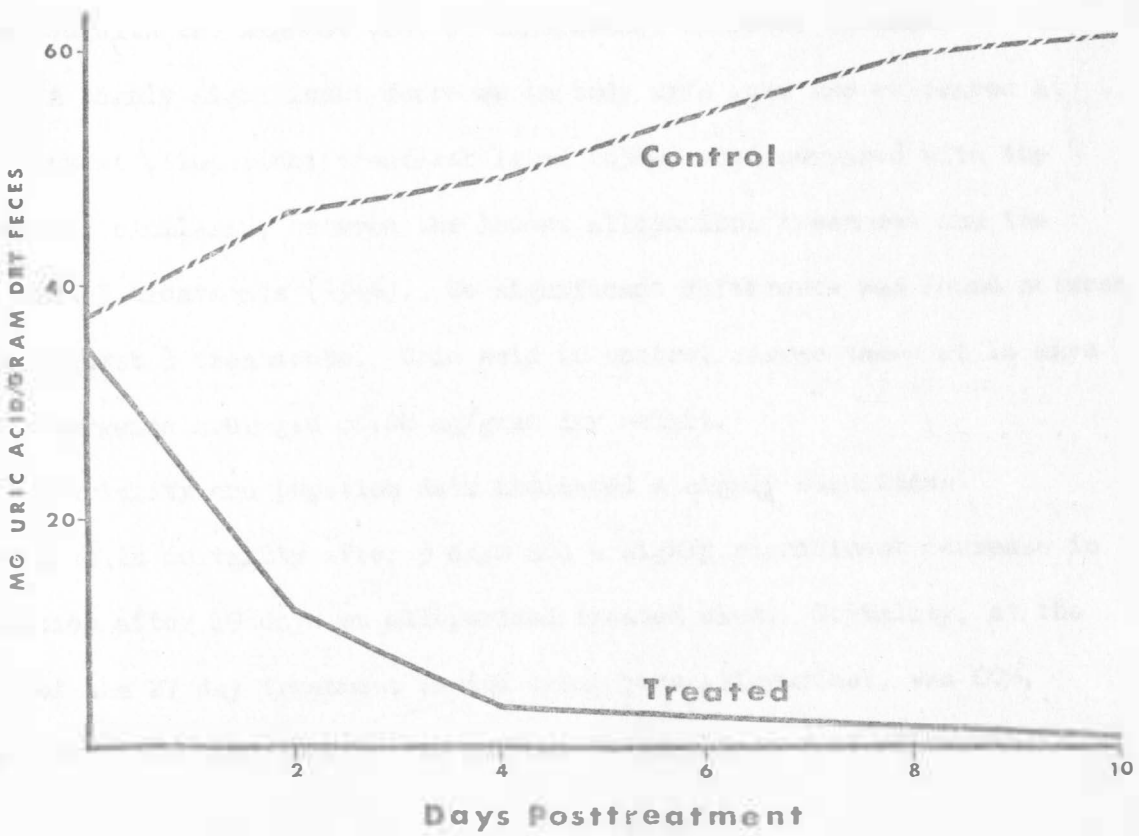
Third-instar nymphs fed 6,476 ppm allopurinol treated diet for 31 days suffered 70% mortality. At 31 days the remaining nymphs were weighed (average 3.52 grams wet and 1.03 grams dry weight/10 nymphs) and compared to an equal number of controls (average 6.54 grams wet and 1.99 grams dry weight/10 nymphs). A difference of 48.24% in dry weights was found. Average uric acid in homogenates of treated nymphs (0.75 mg/gram dry weight) and control nymphs (3.90 mg/gram dry weight) differed by 80.76%. Very few of the treated nymphs reached the fourth instar.

Figure 4. Body uric acid levels of adult grasshoppers treated for 10 days with various doses of allopurinol plus a control.

1. 25,000 ppm allopurinol
2. 15,000 ppm allopurinol
3. 10,000 ppm allopurinol
4. 5,000 ppm allopurinol
5. none (control)

Figure 5. Feces uric acid levels of adult grasshoppers treated for 10 days with various doses of allopurinol plus a control. Curve for treatments is for averaged data from all dose levels.


MG URIC ACID/GRAM DRIED BODY
3
2
1
5
4
2
1
3
2
4
6
8
10
Days Posttreatment


MG URIC ACID/GRAM DRY FECES
60
40
20
Control
Treated
2
4
6
8
10
Days Posttreatment

Thirty percent of those that died were in the process of molting.

Fecal uric acid of treated nymphs (first and third instar) decreased 85% or more during their respective treatment periods while control levels increased 12.37%.

Experiment C and D.--Armyworm larvae were fed artificial diet containing 4 treatment levels of allopurinol obtained either from 100 mg tablets or pure powder. There was no significant difference between the 2 forms of allopurinol. There was a highly significant difference between treatment levels.

A highly significant decrease in larval weights occurred after 12 or more days postemergence as the allopurinol dose was increased (Fig. 6A. and 6B.). The dry weights of control larvae and those treated with the highest dose of allopurinol differed by 96+%.

A highly significant decrease in body uric acid was evidenced at the lowest allopurinol treatment level (83+%) when compared with the control; similarly, between the lowest allopurinol treatment and the highest 3 treatments (69+%). No significant difference was found between the highest 3 treatments. Uric acid in control larvae taken at 16 days postemergence averaged 85.08 mg/gram dry weight.

Mortality and pupation data indicated a highly significant increase in mortality after 9 days and a highly significant decrease in pupation after 19 days on allopurinol treated diet. Mortality, at the end of the 27 day treatment period using pure allopurinol, was 60%, 57%, 34%, 31%, and 7% from the highest to lowest dose of allopurinol

and the control respectively. Pupation at the end of the same period was 0, 11%, 61%, 61%, and 93% from the highest to lowest dose of allopurinol and the control respectively.

Larvae surviving 27 days on the highest treatment of allopurinol (40) were weighed (0.06 grams/larvae) and placed on untreated diet. These larvae subsequently had 45% mortality, 27.50% pupation (16 to 24 days after being placed on fresh diet), and 17.50% adult emergence. The latter pupae and adults appeared to be normal except for being smaller than those obtained from untreated larvae.

Experiment E.--Allopurinol caused a highly significant inhibition (approximately 50 to 75%) of armyworm larval growth. The addition of selected oxypurines, purines, and pyrimidines to the diet caused a highly significant increase in larval weight in the absence of allopurinol. The addition of uric acid did cause some increase in weight when compared with controls, but the increase was not significant (Fig. 7). There was no significant difference in larval weights in allopurinol treated larvae with the preceding compounds added, although some increase in growth was noted (Table 2).

Mortality and pupation data at 18 and 24 days postemergence are shown in Table 3.

In vitro experiments.--The linear relationship between the amount of crude grasshopper enzyme extract present and units of enzyme activity is shown in Figure 8.

Specific activities of grasshopper (10 mg protein per ml) and armyworm (15.6 mg protein per ml) extracts were 1.87 and 2.81 units

Table 2.—Larval weights of armyworms (grams/larvae), at 12 and 16 days postemergence, treated at 2 days with allopurinol and other compounds.

Treatment	Allopurinol		No Allopurinol	
	12 days	16 days	12 days	16 days
Inosine	0.0652	0.1350	0.1879	0.4940
Bases	0.0716	0.1375	0.1882	0.4935
Oxypurines	0.0843	0.1525	0.1730	0.4573
Uric Acid	0.0634	0.1209	0.1203	0.3923
Control	0.0613	0.1189	0.1203	0.2921

Table 3.--Mortality and pupation data at 18 and 24 days postemergence of armyworm larvae treated at 2 days with allopurinol (4-HPP) and other compounds.

Treatment	% Mortality				% Pupation			
	4-HPP		No 4-HPP		4-HPP		No 4-HPP	
	Days		Days		Days		Days	
	18	24	18	24	18	24	18	24
Inosine	2	28	4	8	0	22	30	78
Bases	2	24	0	16	0	20	22	82
Oxypurines	2	22	4	12	0	20	22	82
Uric Acid	12	24	0	4	0	20	18	62
Control	10	34	2	18	0	8	16	68

per mg protein respectively. Parzen and Fox (1964) found the specific activity of a crude xanthine oxidase extract, from Drosophila melanogaster, to be 2.508 units per mg protein. The relationship between inhibitor concentration and units of xanthine oxidase activity of grasshopper and armyworm homogenates is shown in Figure 9.

The Michaelis-Menten constant (Km) of grasshopper xanthine oxidase with hypoxanthine as substrate was 2.52×10^{-5}M. The inhibition of xanthine oxidase was intermediate between competitive and noncompetitive (mixed) at an inhibitor concentration of 9×10^{-6}M. The inhibition constant (Ki) calculated from the data for mixed inhibition was 3.95×10^{-5}M.

Electron microscopy.--Malpighian tubules of the grasshopper are elongate hollow tubules that open into the intestine immediately behind the stomach (Patton 1963). Their numbers vary from 200 to 300 and are described as excretory in function (Patton 1963, Wigglesworth 1956).

In low-power cross-section (Fig. 10, 12, and 17) the tubule is observed to consist of a single layer of 4-6 epithelial cells resting on a basement membrane (BM). This membrane has many basal infoldings (Bi, Fig. 17 and 18) which extend $\frac{1}{4}$ to $\frac{1}{2}$ way through the cell. The apical surface (A), surrounding the lumen, has many polypoid processess (pp) containing mitochondria (M) (Fig. 10 and 19). Between the basal and apical borders, the cell proper contains a relatively large spherical nucleus (N) with a well defined nuclear membrane (Fig. 17). The cytoplasm is interspersed with numerous mitochondria, vacuoles (V), and lipid-like bodies (lp). The epithelial cells are separated by

Figure 6. A. Larval wet weights of armyworms at 12 and 16 days postemergence, treated at 2 days with various doses of allopurinol plus a control.

1. 6,476 ppm allopurinol
2. 3.138 " "
3. 1,569 " "
4. 623 " "
5. none (control)

B. Larval dry weights at 16 days postemergence.

Figure 7. Larval weights of armyworms at 12 and 16 days postemergenc treated at 2 days (no allopurinol present) with:

A. Nothing (control)
B. Uric acid
C. Hypoxanthine and xanthine
D. Purine and pyrimidine bases
E. Inosine

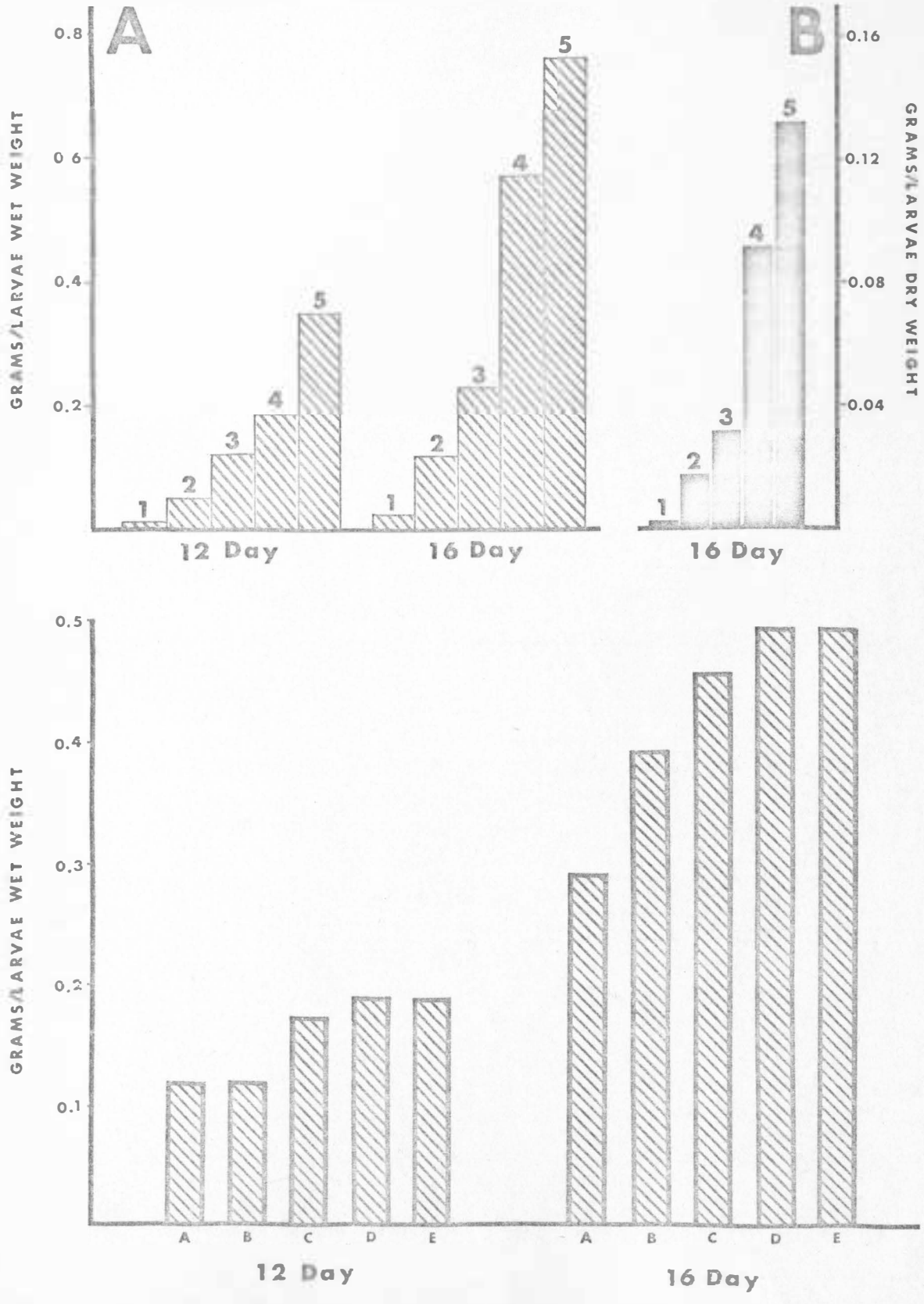
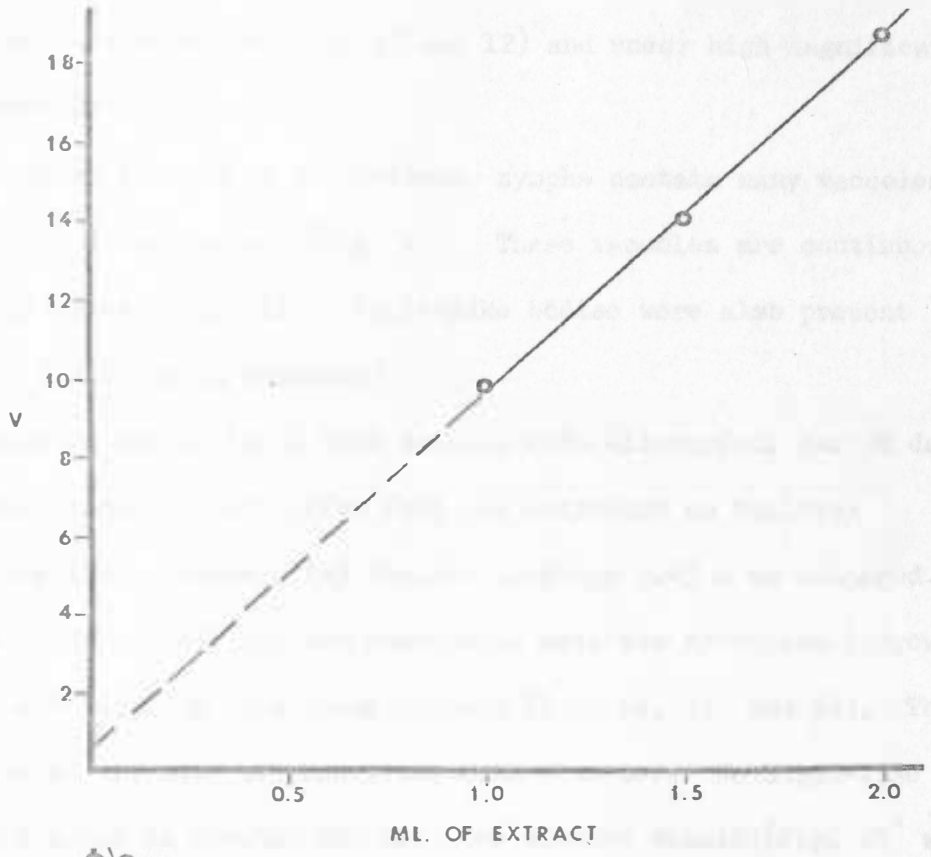
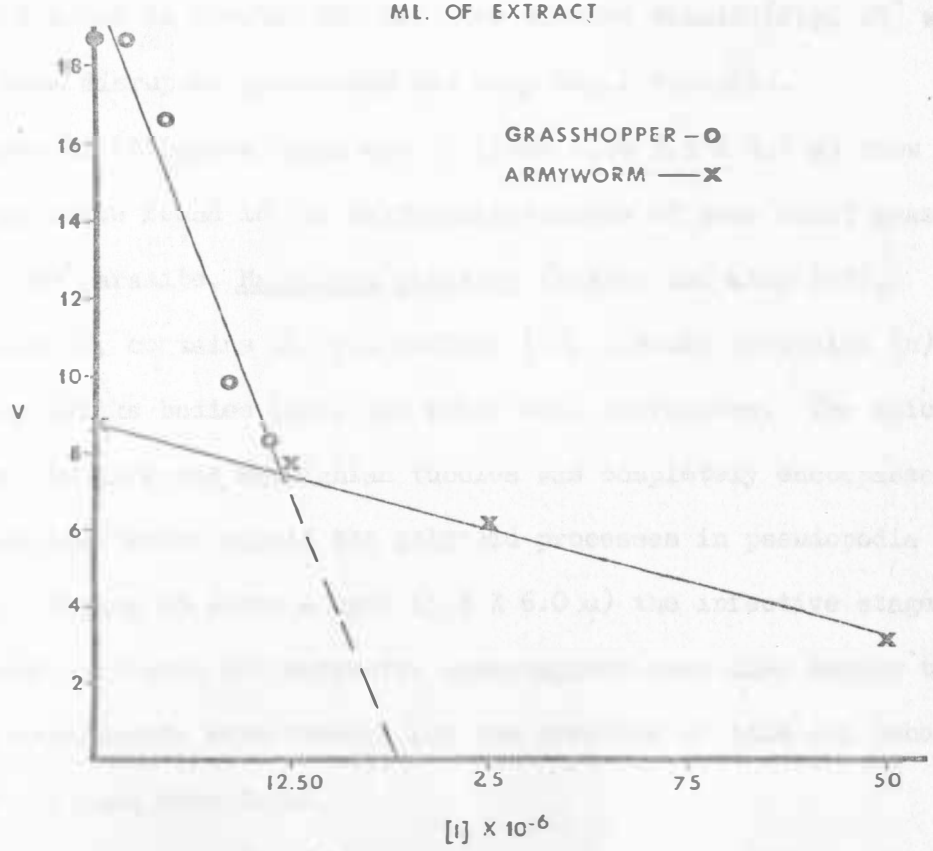

A
B
0.8
0.6
0.4
0.2
0.16
0.12
0.08
0.04
GRAMS/LARVAE WET WEIGHT
GRAMS/LARVAE DRY WEIGHT
1
2
3
4
5
12 Day
16 Day
16 Day
0.5
0.4
0.3
0.2
0.1
GRAMS/LARVAE WET WEIGHT
A
B
C
D
E
12 Day
16 Day

Figure 8. Relationship between the amount of crude grasshopper enzyme extract and velocity of the reaction (V).

Figure 9. Relationship between inhibitor concentration (allopurinol) and units of xanthine oxidase activity (V), using crude grasshopper and armyworm extracts.


18
16
14
12
10
8
6
4
2
V
0.5
1.0
1.5
2.0
ML. OF EXTRACT


GRASSHOPPER — O
ARMYWORM — X
18
16
14
12
10
8
6
4
2
V
12.50
25
75
50
[I] X 10-6

membrane elaborations (cm, Fig. 10 and 12) and under high magnification show crossbridges (Fig. 20).

Malpighian tubules of third-instar nymphs contain many vacuoles with electron dense borders (Fig. 10). These vacuoles are continuous with the cytoplasm (Fig. 11). Lipid-like bodies were also present (average 1.3 X 2.0 μ in diameter).

Tubules of nymphs fed a diet treated with allopurinol for 20 days also contain vacuoles but differ from the untreated as follows: (1) they are less numerous, (2) smaller (average 0.43 μ as compared to 0.88 μ in diameter), (3) not continuous with the cytoplasm (arrows, Fig. 13), and (4) many have dense centers (Fig. 14, 15, and 16). The dense-centered vacuoles averaged 0.46 μ in diameter. No lipid-like bodies were found in treated nymphs. One treated tubule (Fig. 19) was found to have disrupted appearance and many small vacuoles.

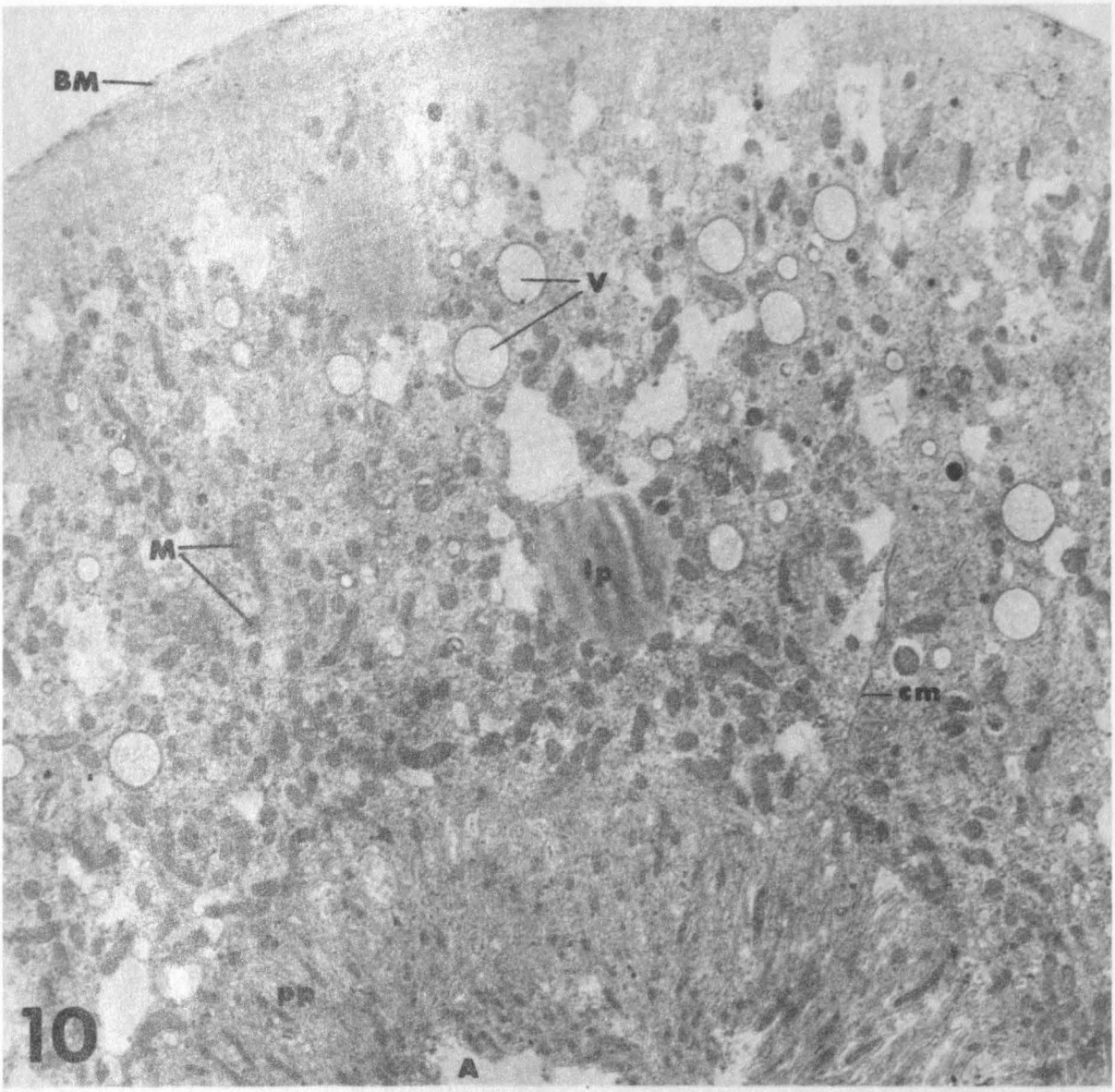
Figures 21 (attached form) and 22 (free form 3.9 X 6.4 μ) show an amoeboid parasite found in the Malpighian tubules of some adult grasshoppers. The parasite, Malamoeba locustae (Taylor and King 1937, Steinhaus 1963), contains an oval nucleus (N), a round nucleolus (n), several lipid-like bodies (lp), and other cell inclusions. The apical surface of the infested Malpighian tubules was completely encompassed by the parasites which engulf the polypoid processes in pseudopodia (Ps) (Fig. 21). Figure 23 shows a cyst (5.2 X 6.0 μ) the infective stage of the parasite. Treated and untreated grasshoppers that died during the preceding experiments were checked for the presence of this and other parasites but none were found.

Plate 1.

Malpighian tubule sections from untreated grasshopper nymphs.

Figure 10. Low-power cross-section of a Malpighian tubule showing positions of cellular elements. The basement membrane (BM) encloses mitochondria (M), vacuoles (V) possibly containing urate material, lipid-like bodies (lp), and a cell membrane (cm). The apical surface (A) is surrounded by polypoid processes (pp), many of which posess mitochondria. X 7,084.

Figure 11. Enlargement of a section of Malpighian tubule showing the vacuoles in greater detail. X 50,000.



10

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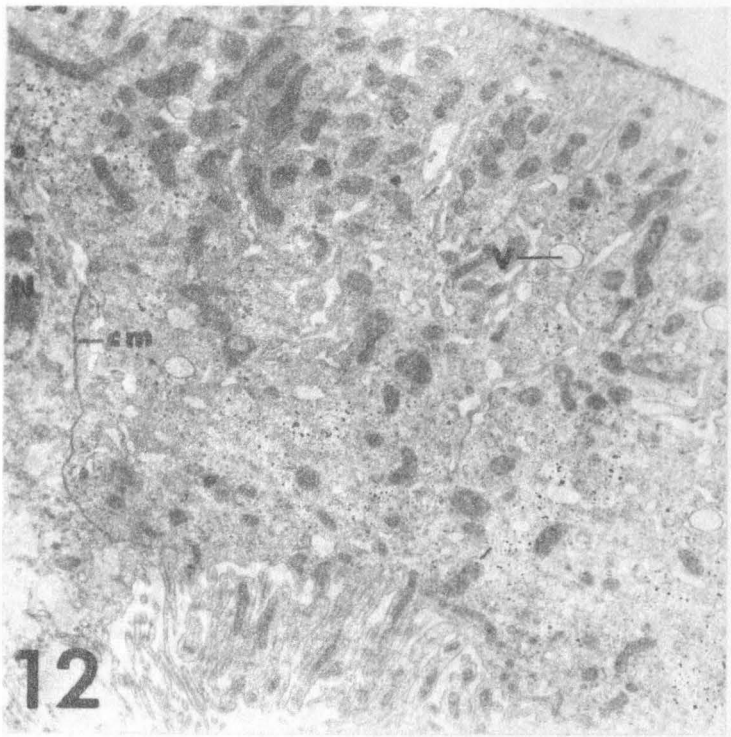
Plate 2.

Malpighian tubule sections from grasshopper nymphs treated with allopurinol.

Figure 12. Low-power cross-section showing several of the small vacuoles typical of Malpighian tubules from treated nymphs. X 6,374.

Figure 13. Enlargement of a section of tubule illustrating the lack of continuity between the cytoplasm and the vacuoles (arrows). X 36,310.

Figure 14, 15, and 16. Dense centered vacuoles found in most treated nymphs. X 13,240, X 36,960, X 84,000 respectively.



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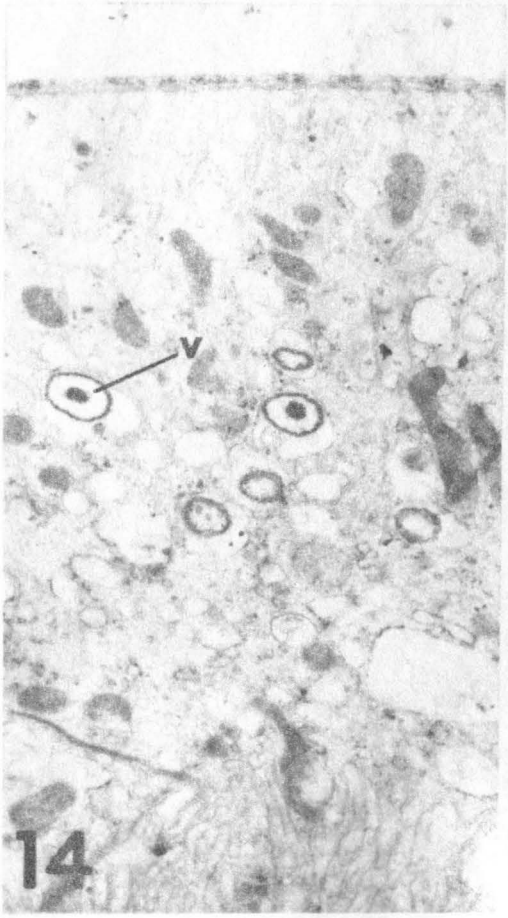

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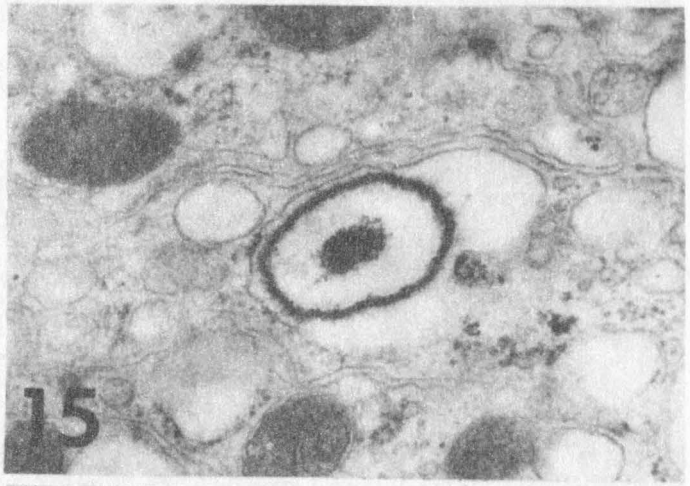

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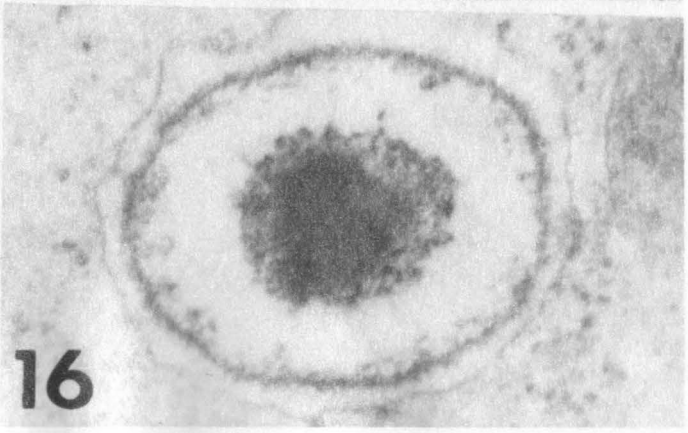


16

Plate 3.

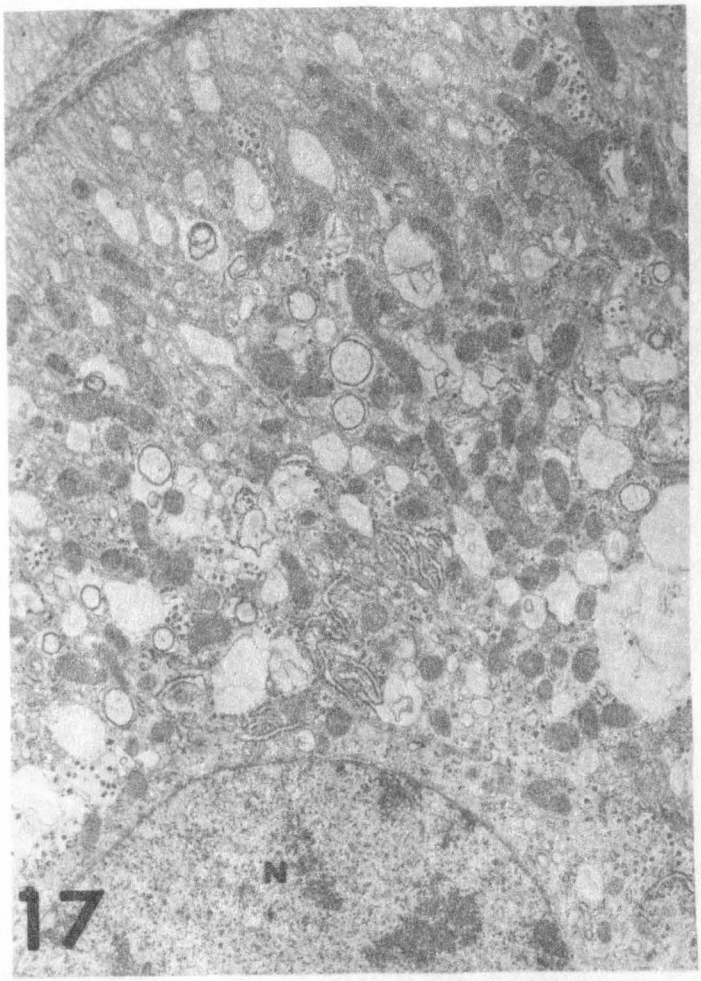
Malpighian tubule sections from grasshopper nymphs treated with allopurinol.

Figure 17. A section of tubule which appeared disrupted and contained many small vacuoles. X 8,937.

Figure 18. Basal infoldings (Bi) are shown, these are believed to greatly enlarge the surface area exposed to body fluids. X 28,290.

Figure 19. An enlargement of the apical surface of a tubule displaying the polypoid processes (pp) filled with elongate mitochondria (M). These processes are closely involved in the secretion and absorption of body metabolites and minerals. Note the small vacuoles within the processes. X 13,240.

Figure 20. Cell membrane elaborations found between adjacent epithelial cells showing many cross bridges. X 50,000.



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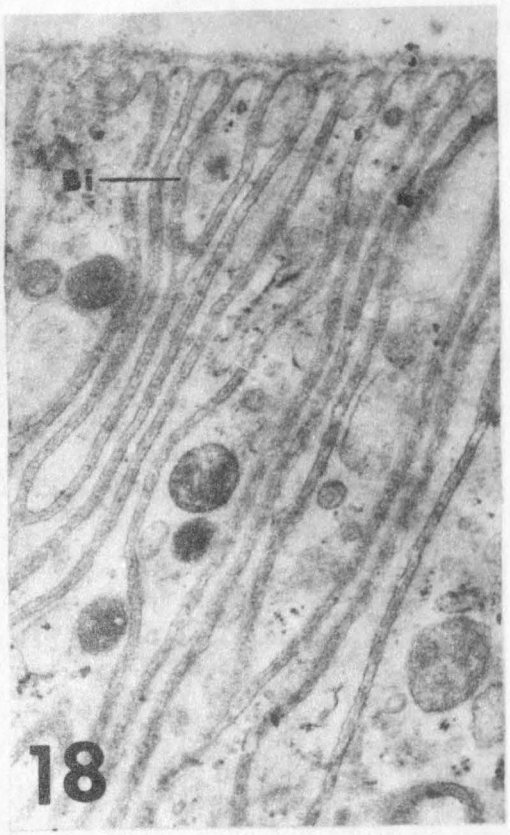

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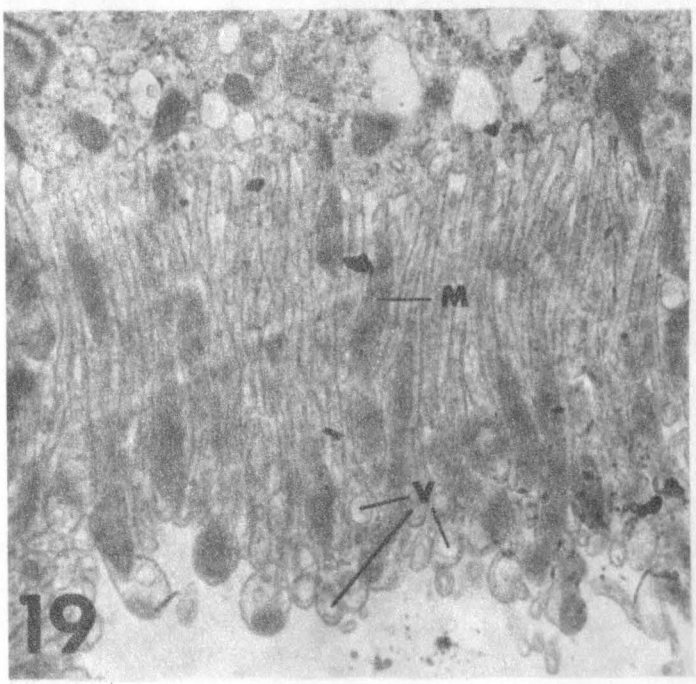

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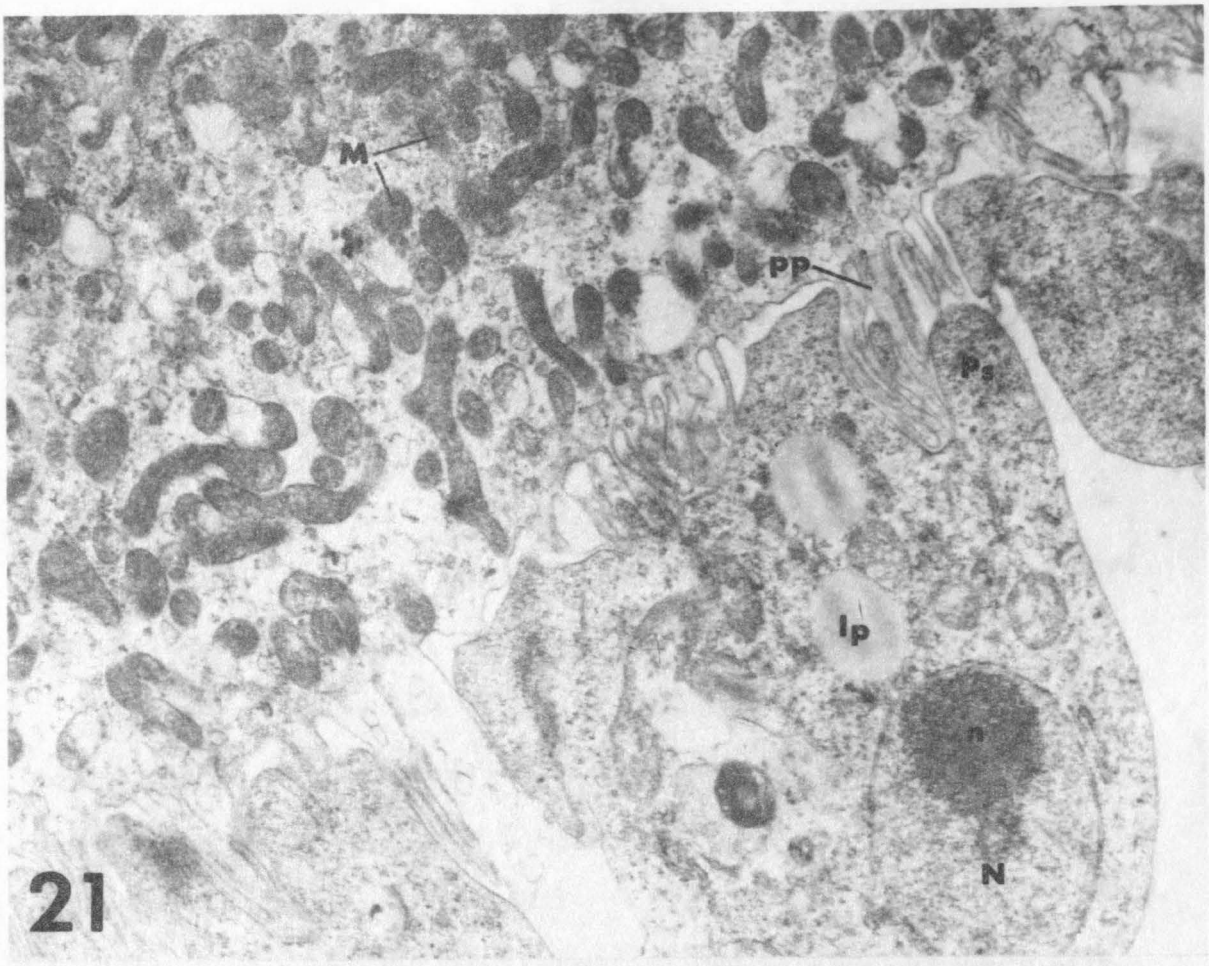
Plate 4.

Sections of Malpighian tubules from adult grasshoppers infested with the parasite Malamoeba locustae.

Figure 21. The amoeboid parasite is shown attached to the polypoid process (pp) of an infested tubule. The parasite contains an oval nucleus (N), a round nucleolus (n), several lipid-like bodies (lp), and other cell inclusions. Note the lack of mitochondria in the polypoid processes. X 16,550.

Figure 22. The free form of the parasite is shown as it appears in the lumen of an infested tubule. X 12,940.

Figure 23. The cyst stage of the parasite, containing lipid-like bodies, was found in large number in the lumen of infested Malpighian tubules. X 13,475.



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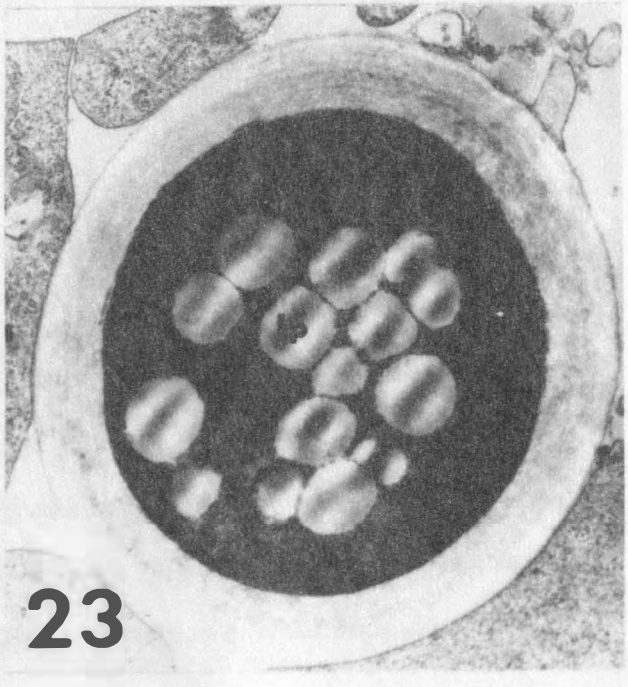


23

DISCUSSION AND CONCLUSIONS

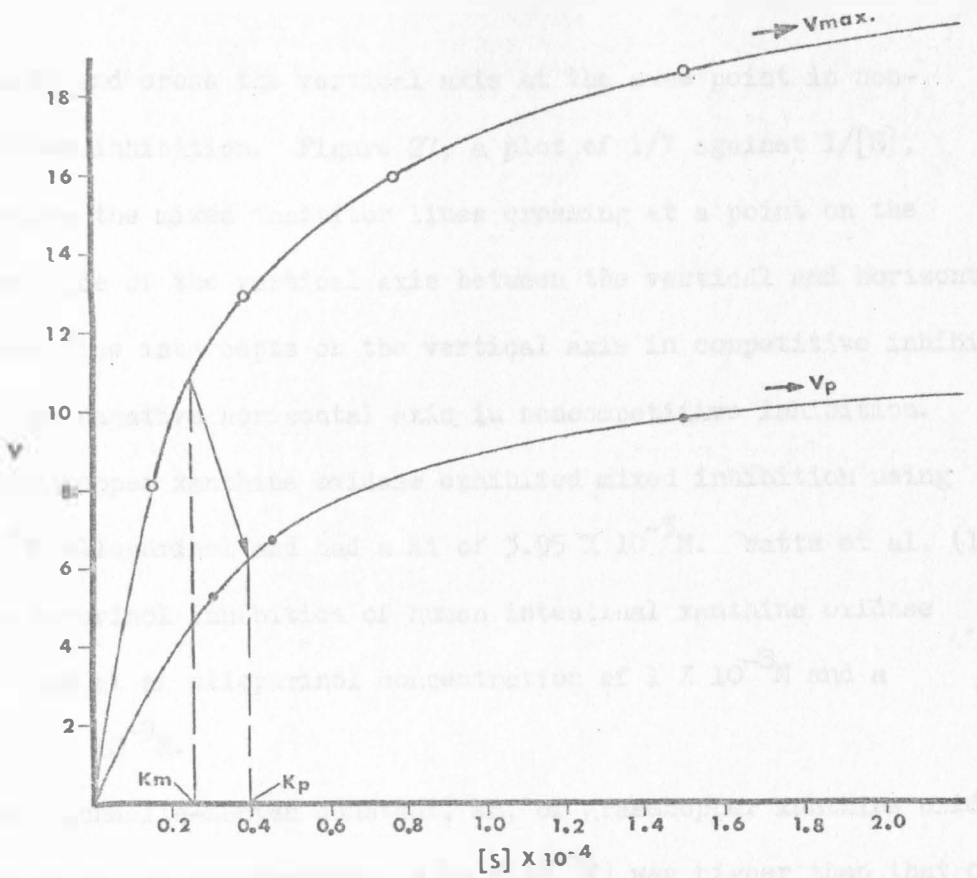
Crude enzyme preparations of grasshopper and armyworm xanthine oxidase were inhibited by allopurinol. A corresponding highly significant decrease in body uric acid occurred in crickets, grasshoppers, and armyworms fed diets containing allopurinol. The fecal uric acid, from grasshoppers fed allopurinol, decreased at highly significant rates while control levels increased at a highly significant rate.

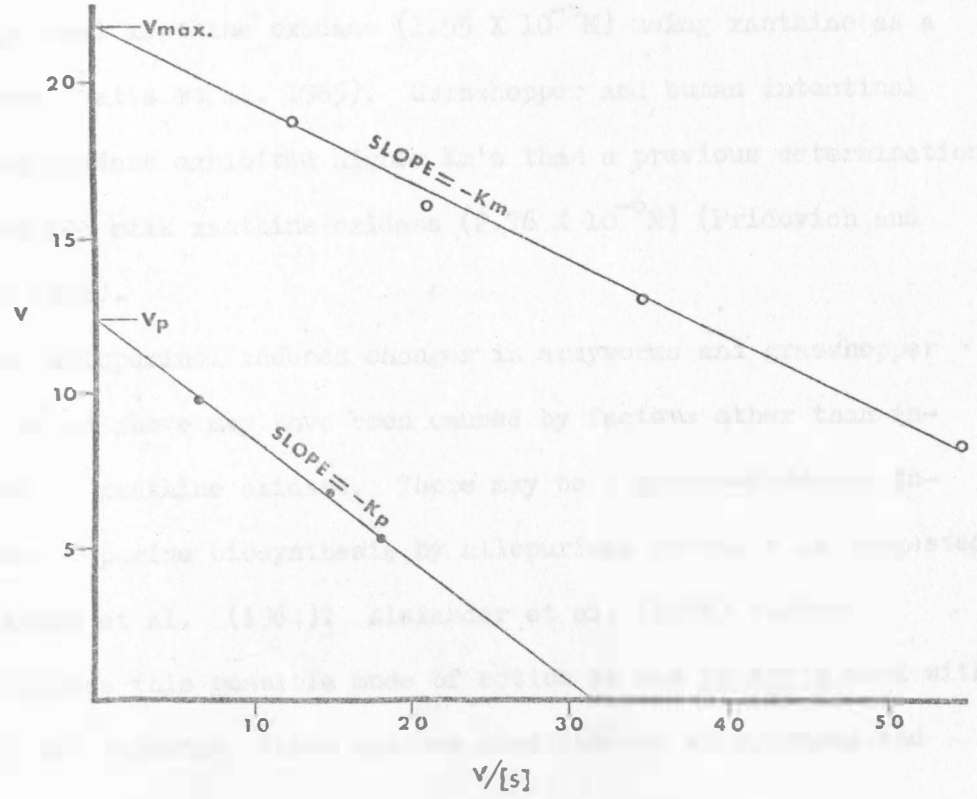
There was a linear relationship between both the amount of crude enzyme present and the concentration of allopurinol added when plotted against units of enzyme activity (Fig. 8 and 9).

Figures 24 - 27 (redrawn from Dixon and Webb 1964 using data for grasshopper xanthine oxidase) illustrate the mixed type of enzyme inhibition present in grasshoppers treated with allopurinol (Table 1). Figure 24, a plot of velocity (V) against substrate concentration ([S]), shows an oblique angle to the horizontal axis of an arrow drawn from $\frac{1}{2}$ Vmax. of the enzyme to $\frac{1}{2}$ Vmax. for the inhibitor; the arrow would have been parallel to the horizontal axis in competitive inhibition and vertical in noncompetitive inhibition. Figure 25, a plot of V against V/[S], demonstrates the slopes of the enzyme and inhibitor diverging from different points on the vertical axis; the slopes diverge from a common point in competitive inhibition and are parallel to one another in noncompetitive inhibition. Figure 26, a plot of [S]/V against [S] shows a situation similar to the preceding figure only the lines cross the horizontal axis at different points on the negative side of the vertical axis; the lines are parallel to each other in competitive

Figure 24. Crude grasshopper xanthine oxidase and enzyme plus inhibitor (allopurinol) plotted velocity against substrate concentration ([S] X 10^{-4} M).

Figure 25. Grasshopper xanthine oxidase plotted V against V/ [S] . This method is claimed by some authors to be better than the Lineweaver and Burk method (Fig. 27) because it gives a more uniform distribution of points.


Vmax.
Vp
V
18
16
14
12
10
8
6
4
2
Km
Kp
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
[S] X 10⁻⁴


Vmax.
SLOPE = -Km
Vp
SLOPE = -Kp
V
20
15
10
5
10
20
30
40
50
V/[S]

inhibition and cross the vertical axis at the same point in non-competitive inhibition. Figure 27, a plot of 1/V against 1/[S], illustrates the mixed inhibitor lines crossing at a point on the negative side of the vertical axis between the vertical and horizontal axis; the line intercepts on the vertical axis in competitive inhibition and on the negative horizontal axis in noncompetitive inhibition.

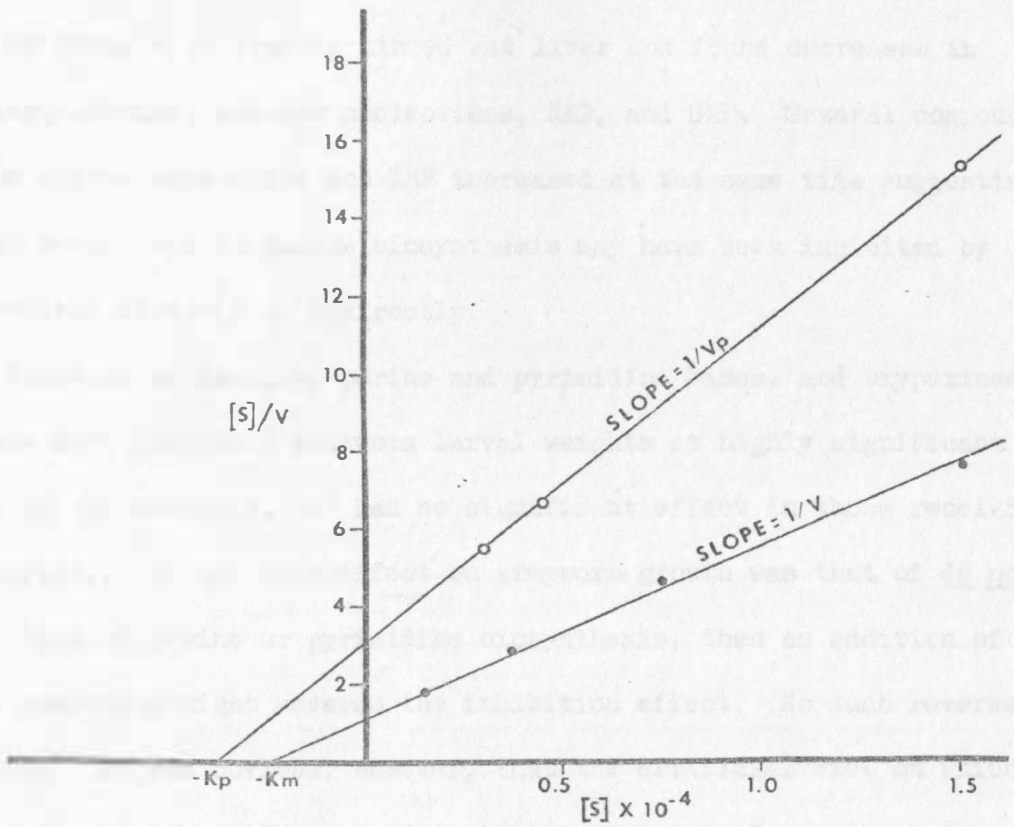
Grasshopper xanthine oxidase exhibited mixed inhibition using 9×10^{-6}M allopurinol and had a Ki of 3.95×10^{-5}M. Watts et al. (1965) found allopurinol inhibition of human intestinal xanthine oxidase competitive at an allopurinol concentration of 1×10^{-8}M and a Ki of 7.6×10^{-9}M.

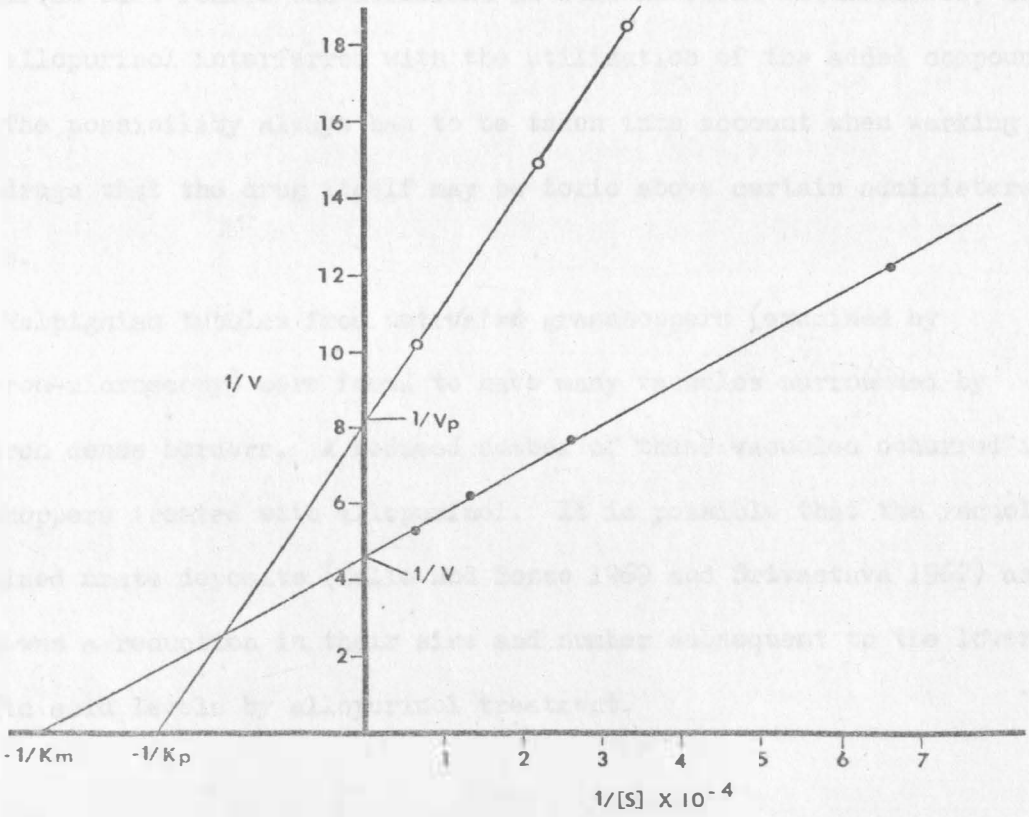
The Michaelis-Menten constant, Km, of grasshopper xanthine oxidase with hypoxanthine as substrate (2.52×10^{-5}M) was higher than that of human jejunal xanthine oxidase (1.55×10^{-5}M) using xanthine as a substrate (Watts et al. 1965). Grasshopper and human intestinal xanthine oxidase exhibited higher Km's than a previous determination reported for milk xanthine oxidase (2.76×10^{-6}M) (Fridovich and Handler 1958).

The allopurinol induced changes in armyworms and grasshopper growth noted above may have been caused by factors other than inhibition of xanthine oxidase. There may be a pseudo-feedback inhibition of purine biosynthesis by allopurinol ribotide as suggested by McCollister et al. (1964). Alexander et al. (1966) further substantiates this possible mode of action in his <u>in vitro</u> work with ^{14}C-labeled formate. These authors administered allopurinol and

Figure 26. This graph illustrates grasshopper xanthine oxidase plotted [S]/V against [S] X 10^{-4} M.

Figure 27. Grasshopper xanthine oxidase plotted by the Lineweaver and Burk method, 1/V against 1/ [S].


18
16
14
12
10
8
6
4
2
[S]/V
SLOPE = 1/Vp
SLOPE = 1/V
-Kp
-Km
0.5
1.0
1.5
[S] X 10⁻⁴


18
16
14
12
10
8
6
4
2
1/V
1/Vp
1/V
-1/Km
-1/Kp
1
2
3
4
5
6
7
1/[S] X 10⁻⁴

labeled formate to freshly minced rat liver and found decreases in inosine, adenine, adenine nucleotides, NAD, and GMP. Several compounds in the citric acid cycle and IMP increased at the same time suggesting one or more steps in purine biosynthesis may have been inhibited by allopurinol directly or indirectly.

Addition of inosine, purine and pyrimidine bases, and oxypurines to this diet increased armyworm larval weights at highly significant rates in the controls, but had no significant effect in those receiving allopurinol. If the main effect on armyworm growth was that of de novo inhibition of purine or pyrimidine biosynthesis, then an addition of these compounds might reverse the inhibition effect. No such reversal occurred. It was obvious, however, that the artificial diet on which the larvae were reared was deficient in some nutrient requirements, and that allopurinol interferred with the utilization of the added compounds.

The possibility always has to be taken into account when working with drugs that the drug itself may be toxic above certain administered levels.

Malpighian tubules from untreated grasshoppers (examined by electron-microscopy) were found to have many vacuoles surrounded by electron dense borders. A reduced number of these vacuoles occurred in grasshoppers treated with allopurinol. It is possible that the vacuoles contained urate deposits (Mello and Bonzo 1969 and Srivastava 1962) as there was a reduction in their size and number subsequent to the lowering of uric acid levels by allopurinol treatment.

SUMMARY

Allopurinol (4-hydroxypyrazolo(3,4-d)pyrimidine) inhibits grasshopper and armyworm xanthine oxidase, lowers their uric acid levels, inhibits metamorphosis, and increases mortality at highly significant rates directly related to increased allopurinol dose. The addition of purine and pyrimidine bases, inosine, oxypurines, or uric acid did not reverse the above effects. The effects can be reversed by discontinuing application of the compound.

The effect of allopurinol on insects needs to be studied in greater detail before conclusions can be drawn as to the use of allopurinol in insect control *per se* or as a synergist with other chemicals. The use of allopurinol as an *in vivo* tool in studying purine catabolism and uric acid excretion in insects may have some possibilities. Implications as to the effects of allopurinol on human systems cannot be drawn from this study but further research would seem to be warranted.

REFERENCES CITED

Alexander, J. A., G. P. Wheeler, D. D. Hill, and H. P. Morris. 1966. Effects of 4-hydroxypyrazolo(3,4-d)pyrimidine upon the catabolism of purines by various tissues of the rat and upon the rate of growth of Morris 5123-C Hepatoma. Biochem. Pharmacol. 15:881-89.

Anderson, A. D., and R. L. Patton. 1955. In vitro studies of uric acid synthesis in insects. J. Exp. Zool. 128:443-51.

Anonymous. 1967. 'Zyloprim' brand allopurinol. A new concept in the therapy of hyperuricemia associated with gout and other conditions. Burroughs Wellcome & Co. (U.S.A.) Inc., Tuckahoe, New York. pp. 31.

Bailey, J. L. 1962. Techniques in portein chemistry. Elsevier Publishing co., New York. pp. 565.

Barrett, F. M., and W. G. Friend. 1970. Uric acid synthesis in Rhodnius prolixus. J. Insect Physiol. 16:121-29.

Cantarow, S., and B. Schepartz. 1967. Biochemistry, 4th ed. W. B. Sanders Co. Philadelphia & London. pp. 898.

Dixon, M., and E. C. Webb. 1964. Enzymes, 2nd ed. Academic Press New York 3, New York. pp. 950.

Elion, G. V., S. Bieber, and G. H. Hitchings. 1954. The fate of 6-mercapopurine in mice. In: Symposium on 6-mercaptopurine. Ann. N. Y. Acad. Sc. 60:297-302.

Elion, G. B., S. W. Callahan, H. Nathan, S. Bieber, R. W. Rundles, and G. H. Hitchings. 1963. Potentiation by inhibition of drug degradation: 6-substituted purines and xanthine oxidase. Biochem. Pharmacol. 12:85-9.

Elion, G. B., S. Callahan, R. W. Rundles, and G. H. Hitchings. 1963. Relationship between metabolic fates and antitumor activities of thiopurines. Cancer Res. 23:1207-13.

Elion, G. B., T. J. Taylor, and G. H. Hitchings. 1964. Binding of substrates and inhibitors to xanthine oxidases from different species. (Abstract). Sixth Int. Congress of Biochem. IV-42. New York.

Falco, E. A., and G. H. Hitchings. 1956. Studies on condensed pyrimidine systems. XV. Some pyrazolo(3,4-d)pyrimidines. J. Am. Chem. Soc. 78:3143-51.

Feigelson, P., J. D. Davison, and R. K. Robins. 1957. Pyrazolopyrimidines as inhibitors and substrates of xanthine oxidase. J. Biol. Chem. 226:993-7.

Fridovich, I., and P. Handler. 1958. Xanthine oxidase. II Studies of the active site. Ibid. 231:899-911.

Hamilton, E. W. 1967. Method of homogenizing insects. J. Econ. Entomol. 60:1461-2.

Horikawa, M., L. L. Ling, and A. S. Fox. 1967. Effects of substrates on gene-controlled enzyme activities in cultured embryonic cells of Drosophila. Genetics 55:569-83.

Irreverre, F., and L. A. Terzian. 1959. Nitrogen partition in excreta of three species of mosquitoes. Science 129:1358-9.

Irzykiewicz, H. 1955. Xanthine oxidase of the clothes moth, Tineola bisselliella, and some other insects. Australian J. Biol. Sci. 8:369-70.

Klinenberg, J. R., S. Goldfinger, J. Miller, and J. E. Seegmiller. 1963. The effectiveness of a xanthine oxidase inhibitor in the treatment of gout (abstract). Arthritis Rheum. 6:779-88.

Klinenberg, J. R., S. Goldfinger, and J. E. Seegmiller. 1965. The effectiveness of the xanthine oxidase inhibitor allopurinol in treatment of gout. Ann. Intern. Med. 62:639-47.

Langenberg, W. G., and H. F. Schroeder. 1970. Visualization of maize dwarf mosaic virus in corn leaf tissue. Phytopath. 60:1299.

McCollister, R. J., W. R. Gilbert Jr., D. M. Ashton, and J. B. Wyngaarden. 1964. Pseudofeedback inhibition of purine synthesis of 6-mercaptopurine ribonucleotide and other purine analogues. J. Biol. Chem. 239:1560-63.

Mello, M. L. S., and L. Bozzo. 1969. Histochemistry, refractometry, and fine structure of excretory globules in larval Malpighian tubules of Melipona quadrifasciata (Hym., Apoidea).

Nation, J. L. 1963. Identification of xanthine in excreta of the greater wax moth, Galleria mellonella (L.). J. Ins. Physiol. 9:195-98.

Nation, J. L., and R. L. Patton. 1961. Nitrogen partition in insect excreta. Ibid. 6:299-308.

Parzen, S. D., and A. S. Fox. 1964. Purification of xanthine dehydrogenase from Drosophila melanogaster. Biochim. Biophys. Acta. 92:465-9.

Patton, R. L. 1963. Introductory insect physiology. W. B. Saunders Co. Philadelphia and London. pp. 245.

Pomales, R., S. Bieber, R. Friedman, and G. H. Hitchings. 1963. Augmentation of the incorporation of hypoxanthine into nucleic acids by the administration of an inhibitor of xanthine oxidase. Biochem. etbiophs. acta 72:119-31.

Praetorius, E., and H. Poulsen. 1953. "Enzymatic determination of uric acid". Scand. J. Clin. & Lab. Invest. 5:273-5.

Robins, R. K. 1956. Potential purine antagonists. I. Synthesis of some 4,6-substituted pyrazolo(3,4-d)pyrimidines. J. Am. Chem. Soc. 78:784-9.

Rockstein, M. 1965. The physiology of insecta. Vol. II. Academic Press, New York and London. pp. 1025.

Rundles, R. W., E. N. Metz, and H. R. Silberman. 1966. Allopurinol in treatment of gout. Ann. Intern. Med. 64:229-58.

Rundles, R. W., H. R. Silberman, G. H. Hitchings, and G. B. Elion. 1964. Effects of xanthine oxidase inhibitor on clinical manifestations and purine metabolism in gout (abstract). Ibid. 60:717-36.

Rundles, R. W., J. B. Wyngaarden, G. H. Hitchings, G. B. Elion, and H. R. Silberman. 1963. Effects of a xanthine oxidase inhibitor on thiopurine metabolism, hyperuricemia and gout. Trans. Ass. Amer. Physicians. 76:126-45.

Sokoloff, Leon. 1957. Symposium on gout: the pathology of gout. Metabolism 6:230-59.

Sorensen, L. B. 1963. Current concepts of gout and its treatment. Med. Clin. Am. 47:169-201.

Srivastava, P. J. 1962. Physiology of excretion in the larva of Corcyra cephalonica (Stainton). J. Ins. Physiol. 8:223-31.

Steel, R. G. D., and J. H. Torrie. 1960. Principles and procedures of statistics. McGraw-Hill Book Co., Inc. New York, Toronto, and London. pp. 481.

Steinhaus, E. A. 1963. Insect pathology an advanced treatise, Vol. 2. Academic Press. New York and London. pp.689.

Sutter, G. R., and E. Miller. 1971. Rearing the army cutworm on an artificial diet. J. Econ. Entomol. (in press).

Taylor, A. B., and R. L. King. 1937. Further studies on the parasitic amoebae found in grasshoppers. Trans. Am. Microscop. Soc. 56:172-4.

Thibodeau, G. A., J. R. Felker, and R. M. Swanson. 1969. Hyperlipemia following allopurinol administration. S. Dak. J. Med. 22:21-3.

Watts, R. W. E., J. B. M. Watts, and J. E. Seegmiller. 1965. Xanthine oxidase activity in human tissue and its inhibition by allopurinol (4-hydroxypyrazolo(3,4-d)pyrimidine). J. Lab. & Clin. Med. 66:688-95.

Webb, J. L. 1963. Enzyme and metabolic inhibitors. Vol. 1. Academic Press. New York and London. pp. 949.

Wigglesworth, V. B. 1956. The principles of insect physiology. Willey, New York. pp. 845.

Wyngaarden, J. B., R. W. Rundles, H. R. Silberman, and S. Hunter. 1963. Control of hyperuricemia with hydroxypyrazolopyrimidine, a purine analogue which inhibits uric acid synthesis. Arthritis & Rheumat. 6:306-17.

Yu, T. F., and A. B. Gutman. 1964. Effect of allopurinol (4-hydroxypyrazolo(3,4-d)pyrimidine) on serum and urinary uric acid in primary and secondary gout. Amer. J. Med. 37:885-93.

APPENDIX

Table 1.--Average uric acid levels[a] of adult grasshoppers (body homogenates) taken at 0, 2, 5, and 10 days posttreatment, treated with allopurinol.

Allopurinol Treatment in ppm	Days 0	2	5	10
25,000	0.675 mg	0.478 mg	0.455 mg	0.245 mg
15,000	0.630 "	0.398 "	0.392 "	0.323 "
10,000	0.640 "	0.472 "	0.402 "	0.194 "
5,000	0.650 "	0.550 "	0.470 "	0.354 "
none	0.705 "	0.618 "	0.550 "	0.555 "

a/ Mg uric acid/0.2 grams of sample.

Table 2.--Average feces uric acid levels[a], at 0, 2, 4, 6, 8, and 10 days posttreatment, of grasshoppers treated with allopurinol.

Allopurinol Treatment in ppm	Days 0	2	4	6	8	10
25,000	7.03 mg	2.73 mg	1.13 mg	0.98 mg	0.60 mg	0.33 mg
15,000	6.25 "	2.00 "	0.35 "	0.23 "	0.20 "	0.28 "
10,000	7.40 "	2.15 "	0.60 "	0.58 "	0.53 "	0.30 "
5,000	6.93 "	2.58 "	0.53 "	0.50 "	0.50 "	0.38 "
none	7.50 "	9.32 "	9.87 "	10.97 "	11.92 "	12.20 "

a/ Mg uric acid/0.2 grams of sample.

Table 3.--Larval wet weights (WW) at 12 days postemergence, larval wet and dry weights (DW) at 16 days postemergence, and uric acid levels at 16 days postemergence, of armyworms treated at 2 days with allopurinol in tablet form.

Allopurinol Dose	WW-12 Days[a]	WW-16 Days[b]	DW-16 Days[b]	Uric Acid[c]
6,476 ppm	0.177 gms	0.114 gms	0.015 gms	0.683 mg
3,138 "	0.771 "	0.585 "	0.085 "	0.850 "
1,569 "	1.854 "	1.141 "	0.168 "	0.600 "
623 "	2,835 "	2,853 "	0.458 "	2.017 "
none	5.193 "	3.820 "	0.656 "	17.017 "

a/ Average weight of 3 groups of 15 larvae.
b/ Average weight of 3 groups of 5 larvae.
c/ Mg of uric acid/0.2 grams of sample.

Table 4.--Larval wet weights (WW) at 12 days postemergence, larval wet and dry weights (DW) at 16 days postemergence, and uric acid levels at 16 days postemergence, of armyworms treated at 2 days with allopurinol in pure form.

Allopurinol Dose	WW-12 Days[a]	WW-16 Days[b]	DW-16 Days[b]	Uric Acid[c]
6,476 ppm	0.154 gms	0.134 gms	0.018 gms	0.817 mg
3,138 "	0.494 "	0.485 "	0.046 "	0.850 "
1,569 "	0.878 "	0.988 "	0.141 "	0.867 "
623 "	1.752 "	1.675 "	0.274 "	2.700 "
none	2.696 "	3.489 "	0.568 "	16.733 "

a/ Average weight of 3 groups of 15 larvae.
b/ Average weight of 3 groups of 5 larvae.
c/ Mg of uric acid/0.2 grams of sample.

Table 5.—Percent mortality and pupation of armyworms treated with varying doses of allopurinol in the form of ground tablets.

Allopurinol Dose in ppm	Days Posttreatment 10	15	20	24	27
6,476	27/0[a]	44/0	52/0	68/0	90/0
3,138	19/0	28/0	53/0	71/3	80/7
1,569	22/0	30/0	44/0	57/31	66/31
623	8/0	8/0	22/35	35/60	40/60
none	9/0	9/3	10/85	10/90	10/90

a/ Mortality/pupation

Table 6.—Percent mortality and pupation of armyworms treated with varying doses of pure allopurinol.

Allopurinol Dose in ppm	Days Posttreatment 10	15	20	24	27
6,476	31/0[a]	42/0	51/0	53/0	61/0
3,138	12/0	21/0	34/1	49/5	57/11
1,569	14/0	16/0	22/20	33/54	34/61
623	11/0	13/0	18/35	25/56	31/61
none	4/0	6/3	7/88	7/93	7/93

a/ Mortality/pupation